DOCTORAL THESIS

Haematological and biochemical markers of immune function and iron status in elite athletes during different training periods

Canetti, Elisa

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Haematological and biochemical markers of immune function and iron status in elite athletes during different training periods

by

Elisa Fontenelle Dumans Canetti
BPhty, MSc (High Performance Science)

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Faculty of Health Sciences and Medicine
Bond University

Dr. Bon Gray
(Principal Supervisor)

Prof. Gregory Anderson
Dr. Nicola Bullock
Dr. Christopher McLellan
(Associate Supervisors)

“Gold has its uses, but war is won with iron.”

— George R.R. Martin

Abstract

Elite athletes are commonly required to achieve peak performance multiple times throughout a year, not only to fulfil the multi-event calendar but to guarantee selection to the pinnacle of human athletic endeavour: the Olympic Games. Training periodisation allows for the manipulation of training stimuli, such as varying training load (e.g. intensity and volume) and exposure to extreme environmental stress (e.g. hypoxia), demanding of the already highly-adapted elite athlete, further stimulus-specific physical and molecular adaptations. Fatigue and illness may hinder such adaptations and consequently performance outcomes. Iron metabolism and immune function play critical roles in both the physiological and biochemical adaptations to training and in maintaining a disease-free state. Iron is required for adequate erythropoietic function, oxidative metabolism, regulation of gene expression and cellular immune responses. Monitoring haematological and immunological adaptations to both exercise and training is therefore crucial to ensure the performance benefits of each training period.

In order to limit disruption to training, a new, minimally invasive micro-blood collection method was developed and validated for evaluating circulating levels and expression of immune phenotypes and quantifying granulocytic function obtained from capillary blood sample. Adopting such methodology, this thesis investigated the effects of three specific training periods on haematological and immune parameters in athletes participating in a selection process for the 2016 Rio de Janeiro Olympic Games. Seven elite Australian female kayak athletes, all national and many world champions, participated in this longitudinal investigation from April until December 2015. Throughout the year investigated, the athletes underwent three training camps: high-intensity training (HI; n=7), a normobaric hypoxia (through a 'live-high train-low'; LHTL) protocol: 10-12 hours daily normobaric hypoxia - F_iO_2 14.8% equivalent to 3000m; n=6), and high-volume (HV; n=3).

The acute ("exercise") and chronic ("training") stimuli provided by each of the camps, and cumulatively across all three camps, were sufficient to cause significant redistribution of leucocyte sub-populations and variation to the expression of functional-related surface antigen in granulocytes. Longitudinal analysis of the expression of granulocytic surface antigens in resting samples (pre-training) throughout the HI camp demonstrated declines

of up to 30% for CD11b (p=0.008), 26% for CD18 (p=0.003), 13% for CD16 (p<0.001), and 13% for CD66b (p=0.010). The expression of such antigens on the last training day was still below baseline. Significant declines (>60% p<0.05) in stimulated oxidative burst capacity of granulocytes were observed daily. The daily demands of the HI camp perturbed iron homeostasis with a significant increase in serum hepcidin concentration (78% increase; p=0.025 90% CI [52.4,104]), accompanied by declines of 7% in serum iron 90% CI [-28.9, 14.4] and a 20% reduction in transferrin saturation 90% CI [-9.2, 32.4].

The LHTL training period caused significant daily pre- to post-training average declines of 40% in the capacity of circulating granulocytes to ingest *E.coli* (p<0.05) on every testing day. The phagocytic capacity was restored to baseline values after overnight hypoxic exposure. The introduction of the hypoxic challenge, combined with daily iron supplementation (equivalent to 105mg of elemental iron), caused a significant increase in erythropoiesis marked by a 4.4% increase in haemoglobin mass relative to athletes' body weight (p=0.037) 90% CI [2.1,6.5] and a 6.5% increase in the concentration of transferrin in serum (p=0.007) 90% CI [2.4, 11.2]. There were no cumulative effects of training-induced hepcidin up-regulation throughout the LHTL camp.

The HV training camp also caused average daily declines of 40% in circulating $CD3^{+}CD56^{+}$ lymphocytes. The individual variations in the expression of granulocytic functional-related surface antigens, and iron availability highlighted the need for individual monitoring. Granulocytic functions of phagocytosis and stimulated oxidative burst compensated for each other throughout this training camp.

Combined analysis of data from all training camps indicated preferential recruitment of innate immune components during the LHTL camp while the HI and HV camps increased circulating levels of lymphocytes. The HI training camp caused a greater decrease in granulocytic functional capacity and declines in the expression of functionally-related surface antigens expression than both the LHTL and HV camps.

The findings of this thesis demonstrate that the modulation of immune capacity and iron-related parameters is dependent on the specific training stimulus adopted. Superposition of different training stimuli may limit training adaptation through activation of opposing signalling pathways. Further, the simultaneous introduction of normobaric hypoxia and

iron supplementation to training partially suppressed resting serum levels of hepcidin. The up-regulation of hepcidin as a response to the training-induced inflammation may limit the haematological responses to LHTL, suggesting that a less intense training stimulus be adopted during periods of LHTL. Findings from this thesis provide new insights for the interactions between iron availability, inflammation and hypoxia within the pathways regulating hepcidin expression.

Declaration

"This thesis is submitted to Bond University in fulfilment of the requirements of the degree of Doctor of Philosophy. This thesis represents my own original work towards this research degree and contains no material which has been previously submitted for a degree or diploma at this University or any other institution, except where due acknowledgement is made."

Maintaining the testing standards of the Australian Institute of Sport, the tests specified below were performed by Dr. Nicola Bullock (AIS Senior Physiologist):

- Anthropometric data (i.e. body mass, height and ∑7skinfold – triceps, biceps, subscapular, supraspinale, abdominal, front thigh and medial calf)
- Haemoglobin mass testing
- On-water step test
- Laboratory-based step test

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Elisa Fontenelle Dumans Canetti

Ethics Declaration

The research associated with this thesis received ethics approval from the Bond University Human Research Ethics Committee. Ethics application number RO1721.

Copyright Declaration

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“It is not the mountain we conquer but ourselves.”

— Sir Edmund Hillary

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Publications and Conference Presentations

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List of Abbreviations

ACD	Anaemia of chronic disease
ADC	Analogue to digital convertors
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AMPK	Adenosine monophosphate kinase
AMS	Athlete management system
APC	Allophycocyanin
APC-Cy™7	APC-cyanine tandem fluorochrome
APC-H7	APC-cyanine tandem fluorochrome
APP	Acute phase proteins
APR	Acute phase reaction
ATP	Adenosine triphosphate
BMP	Bone morphogenic protein
BP	Blood pressure
BPC	Block periodisation concept
BPI	Bactericidal/permeability-increasing protein
C	Complement
Ca^{2+}	Calcium
CD	Cluster of differentiation
CGD	Chronic granulomatous disease
COC	Combined oral contraceptive
CR	Complement receptor
DAG	Diacylglycerol
DC	Dendritic cells
DHR	Dihydrorhodamine
DMT1	Divalent metal-ion transporter 1
DNA	Deoxyribonucleic acid
EC	Endothelium cells
EDTA	Ethylenediaminetetraacetic acid
EE	Ethinyl-estradiol
ELISA	Enzyme-linked immunosorbent assay
ES	Effect size
ETC	Electron transport chain
F	Flow
FAD	Flavin adenine dinucleotide
Fe^{2+}	Ferrous iron
Fe^{3+}	Ferric iron
Fe-S	Iron-sulfur cluster
F_iO_2	Fraction of inspired oxygen
FITC	Fluorescein isothiocyanate
FL	Fluorescence

FMPL	N-formyl-methionyl-leucyl-phenylalanine
FSC	Forward scatter
G-CSF	Granulocyte colony stimulating factor
H_2O_2	Hydrogen peroxide
Hb_{mass}	Haemoglobin mass
HI	High-intensity
HIF	Hypoxia-inducible factor
HIF-2α	Hypoxia-inducible factor 2α
HOCl	Hypochlorous acid
HPA axis	Hypothalamic–pituitary–adrenal axis
HR	Heart rate
HRE	Hypoxia response elements
HRP	Horseradish peroxidase
HV	High-volume
ICAM-1	Intercellular adhesion molecule 1
ID	Iron deficiency
IDA	Iron deficiency anaemia
IFN-γ	Interferon gamma
Ig-A	Immunoglobulin A
Ig-G	Immunoglobulin G
IGF	Insulin-like growth factor
IL	Interleukin
IP3	Inositol triphosphate
IRE	Iron responsive elements
IRP	Iron regulatory proteins
JAK	Janus kinase
K	Kayak
LAD	Leucocyte adhesion deficiency
LF	Lactoferrin
LHTL	Live-high, train-low
LO·	Lipid alkyl radical
LOO·	Lipid alkoxyl radical
LPS	Lipopolysaccharide
LT	Lactate Threshold
mAbs	Monoclonal antibodies
MANOVA	Multivariate analysis of variance
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
MPO	Myeloperoxidase
mRNA	Messenger RNA
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NET	Neutrophils release extracellular traps
NF-κB	Nuclear factor κ B

NGAL	Neutrophil gelatinase-associated lipocalin
NH_4^+	Ammonium
NH_4Cl	Ammonium chloride
NK lymphocytes	Natural killer lymphocytes
NRS	Nitrogen reactive species
O_2	Singlet oxygen
O_2^-	Superoxide anion
OC	Oral contraceptive
OH·	Hydroxyl radical
OSA	Obstructive sleep apnea
PAF	Platelet activating factor
PAMPs	Pathogen-associated molecular patterns
PCr	Phosphocreatine
PE	R-phycoerythrin
PE-Cy7	PE-cyanine tandem fluorochrome
PerCP-Cy™5.5	PerCP-cyanine tandem fluorochrome
PGRP	Peptidoglycan recognition proteins
PHD	Prolyl hydroxylase domain
PIP2	Inositol 4,5-biphosphate
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear neutrophilic leucocytes
PMT	Photomultiplier tubes
PNC	Platelet-neutrophil complex
PO_2	Partial pressure of oxygen
PRR	Pattern-recognition receptors
R	Resistance
RDW	Red cell distribution width
RES	Reticuloendothelial system
RM	Repetition maximum
RNA	Ribonucleic acid
RPE	Rating of perceived exertion
rpm	Revolutions per minute
ROS	Reactive oxygen species
SMAD pathway	S mothers against decapentaplegic pathway
SR	Stroke rate
SSC	Side scatter
STAT	Signal transducers and activators of transcription
sTfR	Soluble transferrin receptor
TCA	Tricarboxylic acid cycle
TfR	Transferrin receptor
TGF-β	Transforming growth factor β
TIBC	Total iron binding capacity
TMB	Tetramethylbenzidine
TNF-α	Tumour necrosis factor alpha
TSAT	Transferrin saturation

UIBC	Unsaturated iron binding capacity
URS	Upper respiratory symptoms
URTI	Upper respiratory tract illnesses
VEGF	Vascular endothelial growth factor
VHL	Von Hippel-Lindau
$\dot{V}O_2$	Oxygen consumption
$\dot{V}O_{2max}$	Maximal oxygen consumption
$\dot{V}O_{2peak}$	Peak oxygen consumption
VT	Ventilatory threshold

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Chapter 1

Introduction

Maximal performance is the ultimate goal of any elite athlete, regardless of the sport in which they participate. Excellence in competition is a multi-factorial product, comprising genetic endowment, nutritional status, training and health (Smith, 2003). Fatigue and illness are the most common causes of under-performance in elite athletes (Budgett, 1998). There is a close correlation between training load and both fatigue and illness (Fricker, 1997; Reid, 2004). In an assessment of 41 elite athletes (35 participating in endurance sports) Reid (2004) found that 86% of the endurance sports athletes reported fatigue and 55% of all the assessed athletes experienced recurrent infections, which the author attributed to immunodeficiency, acute or unresolved infections and nutritional deficiency. Gleeson (2007) confirmed that the immune dysfunction caused by exercise is sufficient to increase the athlete's risk of contracting common infections such as those of the upper respiratory tract illnesses (URTI) and influenza.

In preparation for competition, athletes undergo periodised training programs encompassing distinct training blocks of specific durations and intensities, as well as multiple training sessions per day. Such training schedules impose varied strains on all physiological systems, including both the immune system and iron homeostasis. The relationship between exercise intensity and susceptibility to infection displays a "J curve" relationship, where prolonged strenuous exercise impairs immune function (Gleeson, 2007; Nieman, 1994). Prolonged bouts of strenuous exercise can cause temporary immune depression lasting from 3 to 24 hours post-exercise (Fricker et al., 2005; Gleeson, 2006, 2007; Pedersen & Hoffman-Goetz, 2000; Walsh et al., 2011). Continuous, prolonged (>1.5h), moderate (>55% $\dot{V}O_{2max}$) to high intensity exercise (>85% $\dot{V}O_{2max}$) has been shown to cause a more pronounced immune dysfunction when compared to regular moderate exercise (Gleeson, 2007; Pedersen & Hoffman-Goetz, 2000; Walsh et al., 2011). Periods of intensified training, such as the over-reaching phase, may result in a longer lasting immune suppression (Papacosta, 2013).

Optimal cellular proliferation and functioning of the immune system requires availability of micronutrients, particularly iron. Iron is considered one of the most critical micronutrients in the field of exercise nutrition (Heikkinen, Alaranta, Helenius, & Vasankari, 2011). As part of haem, iron is a structural component of myoglobin and haemoglobin molecules and plays an important role as a component within the electron transport chain (ETC). Iron is also essential for immune function as it is a co-factor in lymphocyte proliferation, a structural component of neutrophilic granule contents (e.g.

haem-containing enzymes), and a catalyst for the production of reactive oxygen species (ROS). The role of iron in immune function is made evident as its deficiency may lead to decreases in phagocytosis and oxidative burst capacity - both essential microbicidal processes (Dallman, 1987; Walter, Arredondo, Arévalo, & Stekel, 1986).

Exercise has also been shown to perturb iron homeostasis, increasing research interest in its influence on athletic performance. It is well established that in tissue iron deficiency (ID) iron's basic metabolism and availability are altered. In such situations, concentrations of myoglobin may decrease up to 60%, limiting oxygen (O_2) storage and distribution within the muscle (Garry & Mammen, 2007), and consequently hindering performance (Beard & Tobin, 2000; Beaton, Corey, & Steele, 1989). The incidence of ID in athletes is reported to range from 25 to 36% in non-competition season and can reach up to 70% in competition (Di Santolo, Stel, Banfi, Gonano, & Cauci, 2008; Malczewska, Blach, & Stupnicki, 2000; Reinke et al., 2012). Iron stores are often found to be depleted after a prolonged training phase, usually held prior to competition (Auersperger et al., 2013). Furthermore, iron levels have been shown to take more than 10 days to be restored to baseline values or even remain depleted in some athletes, as Reinke et al. (2012) observed in 14% of the 30 elite athletes (20 rowers and 10 professional soccer players) examined. Several studies have also demonstrated that conditions such as viral infection and ID are more common in athletes when compared to the general population (Nieman, 1997b; Peters & Bateman, 1983; Sinclair & Hinton, 2005; Weaver & Rajaram, 1992). This indicates the need for constant monitoring of biological markers, such as parameters of iron metabolism and immune function in elite athletes, as already acknowledged by many researchers (Fallon, 2004, 2008; Olsson, Eriksson, Ritter, & Heedman, 1984).

Here it is proposed that post-exercise adaptations may alter iron status in elite athletes and be associated with their immunological capability. Also, as training regimes consist of different training periods to accommodate multiple competitions per year, this thesis sets out to identify how these affect biochemical and immunological homeostasis. This thesis hypothesises that periods of intense training will have a greater impact on immune function and iron status compared with less intense training periods. Finally, it is hypothesised that changes in iron availability and iron-binding proteins could potentially be markers for altered immune function in athletes, therefore enabling coaches to alter training loads to prevent illness and consequently avoid decreases in performance prior to competition.

It is now clear, for all the above mentioned reasons, that close monitoring of immune function and iron is indispensable in the quest for optimal sporting performance. The vast majority of the markers available to identify decrements (or alterations) in either immune function or iron status are blood-borne. Further, most of the commercial tests available rely on blood obtained intravenously, as most reference ranges have been set using this collection methodology. Venous blood collection in a sporting scenario, however, is not ideal as it disrupts training, may cause discomfort, has a non-weight baring criteria on the accessed location to avoid soreness and haematoma formation, requires a trained phlebotomist, increases risk of needle stick injuries and generates biological waste. Capillary blood collection has been suggested as an alternative to venous blood collection and is commonly used for sport related parameters such as lactate, blood glucose, and bicarbonate. Immunological parameters, however, pose a challenge to collection site specificity as most of immune events are regulated by membrane surface receptors. Physiological changes in diameter of the vessels as well as blood flow and pressure in the vascular tree, lead to haemodynamic forces that may alter the expression of such surface receptors. As vascular location may influence cell and membrane structures suggestion/usage of an alternative blood collection site warrants further investigation. Therefore, this thesis initially sets out to explore a possible alternative blood collection site that is more suited for monitoring immunological parameters in the elite athletic population. Based on vascular physics and haemodynamics, it is hypothesized that cellular immunological parameters from blood obtained from venous sites will differ from that of blood obtained from a capillary site. Specifically, the knowledge of mechanisms that may account for these site-specific inconsistencies will allow a better educated usage of different sampling sites for blood collection.

1.1) Research Purpose

Training modalities influence both immune function and iron metabolism. Few studies, however, have linked the three. There is abundant evidence to demonstrate the influence of iron metabolism on immune function (Cherayil, 2011; Dallman, 1987; Kemp, 1993; Mullick, Rusia, Sikka, & Faridi, 2006; Omara & Blakley, 1994; Oppenheimer, 2001; Ward et al., 2011). Such studies were mostly performed in iron deficient populations

and/or children, who have different dietary habits and imposed stressors to those of elite athletes.

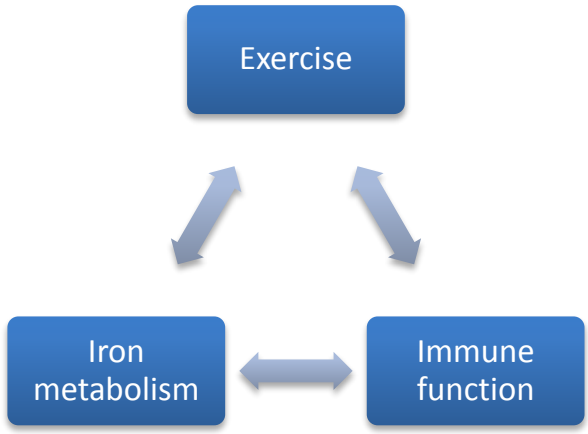
While previous studies have shown that ID negatively affects performance, longitudinal studies monitoring iron status, immune function and athletic performance involving the same group of athletes throughout a season are scarce. Further, studies accompanying an Olympic team selection throughout a training year are even rarer. Previous studies have focused on the competition season despite current training methods having developed into a periodised approach in order to accommodate a multi-competition year. Therefore, training modalities such as high-intensity (HI) and high-volume (HV) training are demanded from athletes at multiple times throughout the year in short period blocks. Such schedules may not allow for proper recovery of baseline iron values (Auersperger et al., 2012; Wilkinson, Martin, Adams, & Liebman, 2002) and rest periods of up to 10 days may be insufficient to achieve this. It is known that ID occurs in stages; therefore, inadequate recovery may further deplete iron stores. This highlights the importance of monitoring the responses of these athletes to all the different training regimens imposed throughout the entirety of a season, through a feasible, athlete-friendly approach.

There is a general consensus among sports scientists that, despite having an iron intake in accordance with the recommended daily allowance, athletes still demonstrate decreased baseline iron status when compared to the general population (Schumacher, Schmid, König, & Berg, 2002). Such agreement coincides with the variable effects of iron supplementation on iron status and performance in elite athletes. Progressive iron depletion does not derive from poor iron absorption in the duodenum, but from the inability of the absorbed iron to enter into the circulation (Karl et al., 2010). Thus, modifying training may be more effective than iron supplementation alone in maintaining proper iron stores. To do so, it is imperative to properly monitor iron status fluctuation throughout different training modalities.

The three components on which this thesis focuses - exercise, iron status and immune function - are likely interrelated (Figure 1-1). Exercise is associated with an increase in the levels of cytokines such as IL-1, IL-6, IFN-γ, TNF-α, all of which have been demonstrated through mouse models to increase iron uptake and storage into the reticuloendothelial system (RES) (Alvarez-Hernandez, Liceaga, McKay, & Brock, 1989; Laftah et al., 2006; Ludwiczek, Aigner, Theurl, & Weiss, 2003). Many of these cytokines

act upon hepatocytes, modulating the expression of hepcidin, the major iron-regulatory hormone (Lee, Peng, Gelbart, Wang, & Beutler, 2005; Park, Valore, Waring, & Ganz, 2001). Hepcidin is also regulated by iron status and hypoxia. In ID and hypoxia, hepcidin is suppressed to facilitate macrophage iron release and to increase the absorption of dietary iron. However, hepcidin levels are increased by inflammation and infection, limiting iron availability to microorganisms that usually require iron for survival and proliferation. Already showing low baseline iron values often corresponding to levels associated with ID (Beard 2000, Auersperger 2013), iron stores in the athletic population is also challenged by the acute phase response to exercise that mimics iron homeostatic retention in inflammation and infectious scenarios (Gabriel 1997; Fallon 2001). Thus, hepcidin has been proposed as the common factor linking iron, immune function, and exercise. Plasma hepcidin increases post-exercise, decreasing serum iron concentrations, and exacerbating ID in athletes (Auersperger et al., 2013; McClung, 2012; Peeling, 2010). During periods of increased training demand, it is possible that resting serum hepcidin values are increased, further contributing to the decline of available iron. The impact of this continuous decline on iron status should be given more attention, as it may have some correlation with immune suppression often seen in elite athletes.

0**Figure 1-1 –** Interaction between exercise, immune function and iron metabolism



Based upon the evidence presented in the literature, it may be hypothesized that elite athletes enter a cycle where: (1) exercise induces an acute phase response; (2) cytokines are released; (3) cytokines trigger an increase in circulating hepcidin; (4) iron is sequestered inside cells; (5) there is subsequently a decreased availability of iron for myelopoiesis; (6) this results in depressed immune function; leading to (7) persistent fatigue and susceptibility to illness.

The literature strongly supports that intensified training induce a detrimental effect on immune function, however, correlation of these fluctuations post-exercise with clinical outcome requires further investigation (Fricker et al., 2005; Gray, Telford, Collins, Baker, & Weidemann, 1993; Pyne, Smith, Baker, Telford, & Weidemann, 2000). URTI are the illnesses most often studied in athletes, but only a few studies (Cox et al., 2008; Spence et al., 2007) have attempted to correlate infections with the athlete's feedback and/or laboratory confirmation of pathogens. Reduction of the neutrophils' functional capability has been postulated to provide a "window of opportunity" for infectious agents not neutralised by immunoglobulin (i.e. IgA, IgG), to establish an infectious process (Smith & Pyne, 1997). However, studies have shown that exercise also causes a decrease in IgA, particularly salivary IgA (Gleeson & Pyne, 2000), increasing host vulnerability.

Finally, even though previous studies strongly demonstrate the negative effects of exercise on immune function, few have suggested strategies to prevent or reduce the risk of illness. Pyne et al. (2000) have provided guidelines for the maintenance of immunocompetence in athletes. The suggested strategies encompass interventions in training, environmental factors, psychological and behavioural considerations, and clinical and medical components.

Particular attention must be directed to athletes that are more susceptible to iron loss: female athletes, endurance runners and vegetarian athletes (Beard & Tobin, 2000). Beard and Tobin (2000) suggest that women may have an increased prevalence to exercise-related alterations in body iron because of a net negative iron balance. The population considered in this thesis are female kayak athletes who, as part of the periodised training to achieve both the aerobic and anaerobic capacities required for kayaking, take part in a variety of modalities (i.e. running, strength training). Additionally, according to Beard and Tobin (2000), decrements in proliferation and activity of immune effectors are linked to iron deficiency though molecular and cellular mechanisms that still require elucidation. Therefore, the cause-consequence relationship between iron biology and immune function in athletes warrants further investigation.

The objective of this thesis was twofold. First, it aimed to establish a minimally-invasive, athlete-friendly, field-based method of blood collection which allows analysis of iron variables as well as phenotypical identification of leucocyte populations and immune functions. Then, utilizing the developed methodology, this thesis aimed to identify

periods of increased susceptibility to infection based on iron parameters and leucocyte concentration in circulation and function according to the different training loads imposed to elite female kayak athletes in preparation for the 2016 Olympic Games in Rio de Janeiro, Brazil.

1.2) Investigation Design

This investigation set out to provide a detailed analysis of immunological and haematological parameters during different training periods in elite female kayak athletes. Different to most of the studies available, this investigation aimed to produce a "real-life" analysis of the imposed stress of daily training in both iron and immune function in international-level athletes undergoing Olympic selection. Therefore, athletes were kept in their normal training environment with no visit to the exercise laboratory and minimal intervention to their normal training schedules. To do so, the present study sought initially to find a field-based, athlete friendly means of blood collection. The commonly used venous collection to obtain a blood sample is not welcomed by the athletes, particularly in kayaking where upper limb movement is predominant, since post-collection soreness to the arm is experienced often. Furthermore, in a detailed analysis where multiple samples are collected daily, compliance to such a method is diminished.

Hence, the first study of this investigation compared immunological and haematological parameters obtained from a venous blood sampling to two commonly used capillary sampling sites (finger and earlobe). The development and validation of new methodology of blood collection enabled the research to collect up to six samples per training day with 100% compliance of the athletes involved. It must be noted that as data was collected throughout a team selection and Olympic qualifying year, the number of athletes per training camp varied.

Utilizing the methodology described on the first study, the second study followed a two-week HI training camp where athletes trained three times per day. High training loads were imposed throughout this over-reaching training camp.

The third study followed the group of athletes selected by the coaches from the previous camp during a team-specific training camp with an altitude component. Athletes were submitted to two weeks of “live high, train low” (LHTL) methodology by sleeping in hypoxic normobaric tents.

The fourth study observed the same group of athletes during a HV training phase. A decreased training load and the adoption of non-kayak specific modalities during training characterised this accumulation training camp.

Finally, the fifth study integrated findings from the training camps and aimed to identify training periods that cause greater strain on immune function and iron status or do not allow for appropriate recovery from athletes. In doing so, suggestions were made towards tailoring a training year, either by rearranging different training blocks or increasing recovery time between heavy training loads, to decrease the incidence of ID and minimise immunological suppression prior to competitions.

1.3) Research Significance

The significance of this research is manifold. Firstly, this research supports the use of capillary blood sampling in the analysis of leucocyte count and function, providing evidence that such methodology may allow for enhanced monitoring with minimal disruption to training. Increased sampling times may aid in better understanding of how the imposed physical stress impacts immune function. By allowing increased data collection and monitoring, coaches and supporting staff may be able to individualise training and prescribe appropriate, non-detrimental training loads. Secondly, findings of this research contribute to the knowledge of the interaction between training, iron metabolism, and immune capacity, particularly in a cohort of elite, Olympic level, athletes. Here, a new paradigm of the interplay of training-induced activation of signalling pathways influencing iron status and immune competence is proposed in accordance with the training period imposed.

1.4) Thesis Navigation

This thesis has been prepared focusing on the publications arising from the research, presented as individual chapters.

Chapter two provides a review of literature on the three main components assessed: immune system, iron status, and exercise/training. Throughout this chapter the interaction between these three components is highlighted, exposing the gaps found in literature that inspired this research project.

Chapter three describes in detail the methodology used in all studies.

Chapter four covers the first study. This study is a methodological study where an alternative to venous blood collection is presented. When studying exercising subjects, multiple samples may be required through a day as it was in the case of this research project. With advances in technology, minimal amounts of blood are required to perform laboratorial analysis of haematological and biochemical parameters. Hence the usage of venous blood sampling in athletic testing where multiple samples are often obtained per day is becoming unfeasible and unjustifiable. This study explores further applications of the already used capillary collection method. The applicability of this method was presented in the 12th Symposium of International Society of Exercise Immunology in July 2015, published in the European Journal of Applied Physiology (volume 116, issue 8, pages 1583-93 doi: 10.1007/s00421-016-3413-z) and it was adopted throughout the research project.

Chapters five, six and seven analyse the effects of exercise on immunological and iron related parameters in elite female kayak athletes by employing a single group pre-post training period (camp) design in three distinct training camps throughout an Olympic team selection training year. Chapter five focuses on a HI training period where the main physiological outcomes expected are increase in speed endurance and power. Chapter six analyses the introduction of normobaric hypoxia as an additional stressor to their training. Athletes maintained the training intensity as that of study five, but were under the LHTL regime, which will be described in detail throughout this thesis. Chapter seven follows a HV training phase, commonly adopted by athletes throughout a training year.

Chapter eight provides a longitudinal analysis of iron and immune related parameters from blood samples obtained venously prior to and at the end of each of the training camps described above. This chapter also highlights the main findings in each individual camp and draws comparisons between the different training periods.

Chapter nine provides a combined discussion of all the studies. This chapter includes suggestions of practical applications, limitations of this study and future directions.

Chapter 2

Literature Review

Athletic performance is highly dependent on innate physiological capacity and response to training. The ability to achieve optimal performance levels at a specific time demands constant manipulation of the training regime. The main objective of training is to provide the body with a stimulus to adapt to, culminating in the increased capacity of various physiological systems to perform increased workloads (Smith & Roberts, 1994). Adaptation will only occur if fatigue-inducing stimuli of HI or HV exercise are imposed. Recovery periods will then allow the body to restore the disrupted homeostasis. Therefore, as Smith and Roberts (1994) described, "the goal is to induce sufficient fatigue to stimulate the adaptive process, but not to disrupt homeostasis to such a large extent that the body cannot recover."

2.1) Flatwater Sprint Kayak

Included in the Olympics since 1936 (men; 1948 women), flat-water sprint kayak events are raced by men and women and may have one (K1), two (K2) or four (K4) paddlers, racing in 200m, 500m or 1000m (men only) distance events. Unlike the Olympic events, women race at all three distances in World and Australian Championships. Australia has a high world ranking in kayak sprint racing and is usually in the top 5 nations at a World Championships or Olympic Games. The sport has brought a total of 22 Olympic medals to Australia since its athletes first participated at the 1956 Melbourne Olympic Games (Battaglia et al., 2008).

2.1.1) Physiological Demands

Sprint kayak is a highly physiologically and psychologically demanding sport where, from a stationary start in a sitting position, athletes are required to paddle a given distance and cross the finish line as fast as possible. To do so, kayak paddlers rely on high levels of both aerobic capacity and anaerobic power.

The quick-start strategy requires the athletes to exert supra-maximal intensities to ensure advantageous positioning in the beginning of the race – a determinant of race success (Ualí et al., 2012). Such HI short-duration (<6 seconds) exercise requires immediate adenosine triphosphate (ATP) supply, obtained mainly by the hydrolysis of

phosphocreatine (PCr), termed the phosphagen system (Greenhaff & Timmons, 1998; Sahlin, Harris, & Hultman, 1979). This energy system utilises the PCr reserve found in skeletal muscle. PCr, adenosine diphosphate (ADP) and hydrogen ion (H^+) are catalysed by creatine kinase forming ATP and creatine. A second reaction in this system is the formation of ATP and adenosine monophosphate (AMP) from two ADP molecules catalysed by adenylate kinase. Further, AMP is deaminated by AMP deaminase forming inosine monophosphate and ammonium (NH_4^+). Rapid consumption of the PCr reserves renders this pathway unsustainable for periods of longer duration, with reports demonstrating its initial decline after 1.3 seconds of HI contractions (Maughan, Gleeson, & Greenhaff, 1997).

Aiming to maintain maximal velocity throughout the initial splits of the race, ATP production relies on a second anaerobic pathway, glycolysis. This pathway was once thought to initiate only after PCr reserves were depleted. However, studies show that ATP resynthesis from this glycolytic pathway initiates at the onset of exercise, concomitantly to the phosphagen system (Casey, Constantin-Teodosiu, Howell, Hultman, & Greenhaff, 1996; Jones & McCartney, 1986). Through glycolysis, maximal ATP resynthesis rate is reached at 10-15 seconds of exercise (Baker, McCormick, & Robergs, 2010). In glycolysis, ATP in the muscle is resynthesised through the breakdown of muscle glycogen (or free glucose) to pyruvate through a series of chemical reactions, each catalysed by a specific enzyme (reviewed in Dashty, 2013). Increases in concentrations of ADP, AMP (possibly as a by-product of adenylate kinase reaction), inorganic phosphate (P_i) and calcium (Ca^{2+}) in the skeletal muscle activate and up-regulates the activity of the enzyme glycogen phosphorylase which facilitates glycolysis by phosphorolysing glycogen into glucose 1-phosphate (Chasiotis, 1983; Cohen, 1985a, 1985b; Rasmussen, 1986). Subsequent reactions leading to production of pyruvate (and lactate) have been described in detail and reviewed elsewhere (Rogatzki, Ferguson, Goodwin, & Gladden, 2015; Spriet, Howlett, & Heigenhauser, 2000). In HI exercise (and/or altered motor unit recruitment – fast twitch fibres), ATP hydrolysis and accumulation of pyruvate in the cytosol or mitochondria from glycolysis lead to decrease in H^+ consumed by the lactate dehydrogenase reaction or mitochondria, augmenting proton release. Once deemed as the source of muscle fatigue, lactate production is now known to enable the removal of pyruvate, regeneration of NAD^+ and proton buffering thus retarding metabolic acidosis in the muscle during steady-state exercise (Robergs, 2001; Robergs, Ghiasvand, & Parker, 2004). During steady-state exercise, complete

oxidation of pyruvate occurs as it enters the mitochondria and is used in the tricarboxylic acid (TCA) cycle, which will be later described. The fate of the by-products of glycolysis is correlated with exercise intensity (Baker et al., 2010; Spriet et al., 2000).

The women's world record for the high intensity 200m sprint kayak race has been recorded at 37.898s (Carrington, L (NZ) Moscow, Russia 2014), clearly surpassing the time of ATP resynthesis from anaerobic pathways (Gastin, 2001). Therefore, to complete every sprint kayak event, contribution of an aerobic energetic pathway is required. Through mitochondrial respiration, complete oxidation of carbohydrate occurs. The product of glycolysis, pyruvate, is initially broken down into acetyl coenzyme A (acetyl-CoA) through a reaction catalysed by pyruvate dehydrogenase. Combining with oxaloacetate, acetyl-CoA donates its acetyl group and enters a multi-step decarboxylation and dehydrogenation cycle - TCA cycle - where oxaloacetate will be regenerated and enter the cycle again (Krebs, 1954). The H^+ released during the TCA cycle will be transported by reduced NADH and flavin adenine dinucleotide ($FADH_2$) to the ETC. There, a series of haem-containing proteins, cytochromes, perform redox reactions releasing energy to phosphorylate ADP to ATP. Increased metabolic efficiency in this energetic pathway is evidentiated by its yield of a net of 36 ATP from a single molecule of glucose, while glycolysis produces only a net of 2 ATP. In high intensity exercise (non-steady state), cellular ATP demand surpasses ATP supply from mitochondrial respiration, increasing the glycolytic ATP contribution (aerobic-anaerobic transition reviewed in Lucia et al. 1999). If HI exercise is sustained (30s-180s), the rate of glycogenolysis and glycolysis is reduced, further decreasing the rate of production of pyruvate and lactate (Robergs, 2001) . Through the adoption of different training strategies (i.e. endurance, power/sprint training), lactate-proton transport capacity (via proton-linked monocarboxylate transporters) increases, thus retarding muscular acidosis (Juel, 1998; Juel & Halestrap, 1999; Sahlin, 2014). This ensures maintenance of pH enabling optimal function of regulatory enzymes from the above mentioned energetic pathways thus increasing metabolic efficiency.

2.1.2) Training

Metabolic efficiency is a highly trainable component and it is one determinant of performance (Joyner & Coyle, 2008). Reducing O_2 deficit (time taken to reach a steady

state of O_2 consumption to accommodate the demand), through faster oxygen consumption ($\dot{V}O_2$) kinetics, enables a more efficient ATP delivery system (aerobic pathway) as detailed above. Training for aerobic power usually entails high demands on the cardiovascular system, as increases in cardiac output and increasing muscle's ability to extract O_2 from the arterial system (via increased muscle capillaries and/or increased mitochondria concentration) improves $\dot{V}O_{2max}$ (Klausen, Andersen, & Pelle, 1981; Saltin & Calbet, 2006). Further, increasing muscle respiratory capacity, increases lactate threshold allowing exercise to be performed in greater intensities before lactate concentration in blood increases exponentially (Holloszy & Coyle, 1984; Ivy, Withers, Van Handel, Elger, & Costill, 1980).

Byrnes and Kearney (1997) calculated the energy demands of sprint kayaking through laboratory ergometers and demonstrated a 63% anaerobic contribution in the 200m race, 38% in the 500m race and 18% in 1000m race. Bishop (2000) observed that kayak athletes spend the majority of their race around $\dot{V}O_{2peak}$. Through a stepwise multiple regression, Bishop (2000) established that a linear combination of anaerobic threshold and accumulated O_2 deficit accounted for 89% of K500 time. Based on these performance predictors, it becomes evident that both anaerobic and aerobic training are indispensable for a successful event. Thus, the training involves not only kayaking and other specific training activities, but also a multitude of modalities, which include running, swimming and strength training.

The kayak race requires maximum power, acceleration, and speed generation at the starting phase of the race and maintenance of speed throughout the remaining phases. A strong start requires the high power created by the initial paddle in the water to be greater than that of the drag forces (from both water and air) they must overcome. McKean and Burkett (2014) have demonstrated the influence of upper body strength on flat-water sprint kayak performance in elite athletes. In a three-year longitudinal study, these authors showed a strong correlation between strength scores and performance time ($r>0.7$ for both genders in the 500m and 200m), where 1RM bench press increased by 34.8% for men and 42.3% for women while their times to complete 1000m, 500m and 200m were decreased by 4.7% (men) and 4.9% (women), 3.7% (men) and 7.3% (women), and 5.4% (men) and 9.1% (women) respectively. Within the three years, female athletes came within 1.1% of the medallists' times by decreasing their own time by almost 12% in the K1 500-m. The K1 200-m event also showed significant improvement after strength

training with a decrease in time of 6.7%, coming 3.2% of the medallists' time. It has been established that a 0.9% increase in power achieves a 0.3% improvement in time, which is sufficient for a kayak athlete to move into medal contention (Bonetti & Hopkins, 2009).

2.1.2.1) Periodisation

Considering the importance of adaptation to training, a general consensus exists in the literature (Bompa & Haff, 2009; Smith, 2003) that in order to attain a significant improvement in performance, training should follow a cyclic pattern. This is known as periodisation. The traditional periodisation model proposed by Matveyev (1972) utilises regular workload distribution through long periods of time, aiming to develop simultaneously many fitness components (e.g. aerobic capacity, maximum strength) (García-Pallarés, Garcia-Fernandez, Sánchez-Medina, & Izquierdo, 2010; Matveyev, 1972).

Recently, the block periodisation concept (BPC) has been proposed around the basic principle of high concentration of training workload within a given block. These medium-sized training cycles are designated mesocycle blocks. Issurin (2010) identifies three types of mesocycle blocks: accumulation, transmutation, and realisation (Table 2-1). Mesocycle duration is established according to physiological and biochemical prerequisites and usually ranges from 2-4 weeks. A training stage is the combination of each mesocycle and culminates with a specific competition. Correct sequencing of the mesocycles is crucial to competitive performance, and is highly dependent on the sport undertaken and the number of important competitions the athlete will participate in throughout the year.

Table 2-1 – Block periodisation concept (BPC)

Main characteristics	Mesocycle Type		
	Accumulation	**Transmutation**	**Realization**
Targeted motor and technical abilities	Basic abilities: aerobic endurance, muscular strength, basic coordination	Sport-specific abilities: special endurance, strength endurance, proper technique	Integrative preparedness: modelled performance, maximal speed, event specific tactics
Volume-intensity	High volume, reduced intensity	Reduced volume, increased intensity	Low-medium volume, high intensity
Fatigue-restoration	Reasonable restoration to provide morphological adaptation	No possibility to provide full restoration, fatigue accumulated	Full restoration, athletes should be well rested
Follow-up particularities	Monitoring the level of basic abilities	Monitoring the level of sport-specific abilities	Monitoring maximal speed, event specific strategy etc.

Adapted from Issurin (2007)

According to Issurin (2010), the benefits of the BPC as compared with the traditional model (Matveyev, 1972), are the following: (1) the total volume of training can be remarkably reduced, hence reducing the incidence of over-training; (2) the multi-peak training design allows and facilitates successful participation in many competitions over the whole season; (3) monitoring can be more efficient because of the substantial reduction in the number of athletic abilities to be evaluated within each mesocycle; (4) diet and restoration programs can be appropriately modified according to the predominant type of training undertaken; and (5) a multi-stage annual plan creates more favourable conditions for peaking in time for the main competition of the season (Issurin, 2010).

Periods of increased training volume with intensities below that of competition have been suggested to enhance recovery from HI exercise (Seiler, Haugen, & Kuffel, 2007) and increase duration of sustained high muscular power outputs (Coyle, Coggan, Hopper, & Walters, 1988). These notions may have risen from the molecular events triggered by high training volumes, such as the increase in intramuscular calcium (Ca^{2+}). Such increase in Ca^{2+} concentrations activates calcium-calmodulin kinases, which in turn activates peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) (Coffey & Hawley, 2007). PGC-1α has been shown to increase type 1 fibres, mitochondrial biogenesis, fat oxidative capacity and glycogen and GLUT4 concentrations (Adhihetty, Irrcher, Joseph, Ljubicic, & Hood, 2003; Chan & Arany, 2014; Liang & Ward, 2006; Richter & Hargreaves, 2013; Wende et al., 2007). Ingham et al. (2008) showed that elite rowers submitted to 12 weeks of training only below lactate threshold had a significant

improvement in rowing speed at lactate threshold when compared to rowers whose training regime was 30% over and 70% under lactate threshold. Laursen (2010) suggests that these low-intensity HV training periods may enhance the aerobic platform required for specific adaptations that stem from HI training to occur.

Increases in training intensity with reduced training volume aim to enhance intense and prolonged exercise performance (Laursen & Jenkins, 2002). This methodology seeks a functional (short-term) overreaching after which (given appropriate fatigue monitoring and rest), a super-compensatory outcome is expected. In trained athletes (i.e. established endurance base), this transition training has been shown to increase peak power output, fatigue resistance (through increased muscle buffering capacity; Gibala et al., 2006 and phosphocreatine recovery kinetics; Forbes et al., 2008) and muscle oxidative potential while maintaining the athlete's endurance performance (Hawley, Myburgh, Noakes, & Dennis, 1997; Iaia et al., 2009; Iaia et al., 2008; Laursen, 2010). While physiological explanations are still under debate, most authors agree that transition training stimulates skeletal muscle mitochondrial capacity - exemplified by increases in mitochondrial biogenesis regulator PGC-1α (Little, Safdar, Wilkin, Tarnopolsky, & Gibala, 2010) and cytochrome c oxidase activity (Jacobs et al., 2013) -, greater O_2 extraction (via increased capillary network and mitochondrial content (Poole & Mathieu-Costello, 1996), increase in resting muscle glycogen and GLUT 4 protein content (Little et al., 2010). Maintaining the same total work and frequency (3 days/week for 8 weeks), Helgerud et al. (2007) analysed the effects of different aerobic endurance training protocols ((1) long slow distance 70% HR_{max} (2) lactate threshold (85% HR_{max}) (3) 15/15 interval running at 90-95% HR_{max} and (4) 4 minutes running at 90-95% HR_{max} followed by 3 minutes of active resting at 70% HR_{max}) in $\dot{V}O_{2max}$, stroke volume of the heart, blood volume, running economy and lactate threshold. Results indicated that groups 3 and 4 had a significant 5.5% and 7.2% increase on $\dot{V}O_{2max}$, respectively, while no changes were observed for groups 1 and 2 (Helgerud et al., 2007).

Further, periodisation allows for development and training of the different ATP-forming energy systems (Bompa & Haff, 2009). Studies indicate that the recruitment of the different energy systems is not exclusive and that the three work in combination based on ATP requirement – increasing as exercise duration and intensity increase. Researchers have suggested that in a maximum effort exercise of 75 second duration, contribution of both anaerobic and aerobic energy systems is equal. It is then safe to conclude that athletes

in sports requiring an explosive and powerful start and maintenance of work output must train both pathways. One such sport is sprint kayak.

The usual training format for kayak has been described previously (García-Pallarés et al., 2010). Briefly, an initial training phase focuses on developing basic fitness components. In this phase, athletes are submitted to exercises at the second ventilatory threshold (VT2), muscle hypertrophy and technical skills. Evolving from that, a second training phase employs greater aerobic power, usually having athletes train at $\dot{V}O_{2max}$ and maximal muscle strengthening, consequently moving into a more event-specific training. These two main phases are based on the characteristics of kayak described, where the power requirement at the starting portion of the race require greater slow resistance training whereas explosive resistance training enhances force development and speed maintenance (Liow & Hopkins, 2003).

Periodised training has been shown to produce the best performances. Such training usually involves multiple sessions per day emphasizing the development of strength and endurance. Borges et al. (2012) described a season of Olympic kayak training to entail (1) a HV training period – 11 weeks; (2) a HI period - 15 weeks; and (3) a decreased intensity and volume training period, which after seven weeks culminates in the competitive period (Table 2-2).

Table 2-2 – Weekly training performed by kayak athletes during the season leading up to the 2008 Olympics

Training Load	Time point		
	t_1	t_2	t_3
Kayaking (km)	80-140	140-100	100-60
Running (km)	20-35	35-20	20
Swimming (km)	6	6-4	-
Strength (ton)	90-140	140-110	110-100
Calisthenics (min)	120-220	220-140	140-100
Total specific (‘on-water’) training time (min)	90-160	160-70	70-40
Number of weeks	11	15	20

Weekly initial and final values at time points t_1 (January) t_2 (April) t_3 (June).
Adapted from Borges et al. (2012)

Recently, García-Pallarés et al. (2010) compared the use of two training periodisation models on performance markers in elite kayak athletes. Peak oxygen uptake ($\dot{V}O_{2peak}$) and oxygen consumption on second ventilatory threshold ($\dot{V}O_2VT2$) demonstrated similar gains after both traditional and block periodisation. This group argues, however, that block periodisation is more effective since its duration was 10 weeks and 120 hours shorter than the traditional periodisation. In addition, this same group observed a more significant ($p<0.05$) increase in specific abilities (i.e. paddling speed and paddling power) following block periodisation when compared to the traditional periodisation.

Recently it has been described that the training status of the athlete has great influence on their gains for each proposed periodisation. Therefore, more extreme training techniques have been added to the periodisation calendar. One of particular interest is the use of hypoxia.

2.1.3) Altitude Training

2.1.3.1) Physiology of Altitude Training

Part of the concept of training periodisation is the pursuit of physiological adaptations to different stressors that could potentially enhance performance. A common stressor adopted by many coaches is the exposure of the athlete to extreme environments such as high altitude. While the increase in altitude maintains the fractional concentration of inspired O_2 (F_iO_2) at 20.9%, it decreases its partial pressure (PO_2) in proportion to the barometric pressure. Consequently, the amount of O_2 available for delivery to tissue decreases, a concept known as hypobaric hypoxia. As it is unfeasible to transport athletes, training staff and equipment multiples times per year to experience natural hypoxic conditions, man-made altitude chambers (developed by Finnish sport scientists in 1990s) or tents explore the desired adaptations by introducing a normobaric hypoxia, where PO_2 is maintained while reducing F_iO_2 (commonly through nitrogen dilution - addition of nitrogen to ambient air) (Millet, Roels, Schmitt, Woorons, & Richalet, 2010; Wilber, 2001).

In response to acute hypoxia, a compensatory hyper-ventilatory response is initiated, aiming to re-establish homeostasis. Such response, causes a decrease in blood and

alveolar P_{CO2} and, consequently, an alkalinisation of blood. This initial response negatively impacts aerobic capacity within the first 2-3 days of altitude exposure as bicarbonate (natural buffer of H^+) reserves are being released by the kidneys in an attempt to restore blood pH (Goldfarb-Rumyantzev & Alper, 2014; Pinilla, 2014). The immediate response to decreases in PO_2 is quickly countered by long-term adaptive mechanisms (acclimatisation) such as increases in haemoglobin concentration, haematocrit, oxidative enzyme activity, mitochondrial volume, free fatty acid substrate utilisation and capillary density (Bailey & Davies, 1997).

While acute responses to hypoxia result from phosphorylation or redox state of pre-existing proteins, the above mentioned long-term adaptations occur as a result of alterations in gene expression, mediated by the transcriptional regulator hypoxia inducible factor 1 (HIF-1) (Semenza, 2000). The alpha subunit (HIF-1α) regulates HIF-1 biological activity, which includes mRNA and protein expression. In normoxia and adequate iron availability, prolyl hydroxylase domain (PHD) enzymes hydroxylate specific proline residues in HIF-1α (Cockman et al., 2000; Petousi & Robbins, 2014; Semenza, 2004). This permits HIF-1α to be ubiquitinated and to undergo proteasomal degradation via the von Hippel-Lindau tumour suppressor protein (pVHL) binding (Ohh et al., 2000). Hypoxia, however, decreases PHD activity, allowing HIF-1α to accumulate and, when combined with its β subunit, to become transcriptionally active and to bind to specific DNA sequence in hypoxia response elements (HREs) promoter element coupled to target genes (Petousi & Robbins, 2014). These genes include regulators of cell growth, differentiation and death (e.g. insulin-like growth factor (IGF)-2), erythropoiesis (e.g. EPO, transferrin, transferrin receptor, haem oxygenase-1), angiogenesis (e.g. VEGF) and energy metabolism (e.g. glucose transporter-1, -3 and -4, lactate dehydrogenase-1, phosphofructokinase L, pyruvate kinase M) (Favier, Britto, Freyssenet, Bigard, & Benoit, 2015; Hirota & Semenza, 2006; Iyer et al., 1998; Kumar & Choi, 2015; Lee et al., 1997; Manalo et al., 2005; Semenza, 2000). Upregulation of key erythropoietic proteins, glycolytic enzymes and glucose transporters promotes preferential use of glycolytic energetic pathways, possibly enhancing aerobic metabolism (Kumar & Choi, 2015; Semenza, 2004). Combined, these adaptations have been reported to render the advantage sought in performance at sea level (Gore et al., 2001; Levine & Stray-Gundersen, 1997; Saunders et al., 2004; Wilber, Stray-Gundersen, & Levine, 2007).

There is still discussion regarding the best methodology of exposure to normobaric hypoxia. Different protocols, such as long continuous or intermittent exposure seem to benefit more sub-elite athletes and elite athletes (already highly adapted to training stimuli), respectively. Further, protocols utilising natural versus artificial altitude have been shown to enhance performance in both elite and sub-elite athletes (Bonetti & Hopkins, 2009). While acute hypoxia ($\leq$ 90 minutes/day), highlighted in intermittent hypoxic exposure studies, has demonstrated no (Humberstone-Gough et al., 2013; Julian et al., 2004) or a detrimental effect on performance (Hamlin, Marshall, Hellemans, Ainslie, & Anglem, 2010; Lundby, Millet, Calbet, Bartsch, & Subudhi, 2012; Millet et al., 2010), prolonged hypoxic exposure (> 12 hours/day) has been positively correlated with improvements in performance. In highly trained athletes, exposure to such stressor has shown to supplement training as it suggestively augments both aerobic power and anaerobic capacity (buffering capacity; Gore et al. 2001 and lactate metabolism; Nummela and Rusko, 2000) (Mizuno et al., 1990; Saltin et al., 1995).

The most commonly adopted protocol is the "live-high train-low" (LHTL), where athletes train at sea-level and sleep in a normobaric hypoxic environment. The mechanisms responsible for the observed performance improvements in this method are still in debate. Authors highlight that adopting LHTL allows for the previously described desired physiological adaptations to occur without having to decrease training intensity and fatigue and declines in aerobic performance, usually seen in chronic hypoxic exposure (Millet et al., 2010). While it is well established that red blood cell volume increases thus increasing O_2 distribution, some argue that the improvement in power per unit of $\dot{V}O_2$ is more likely to influence performance (Gore & Hopkins, 2005; Schmitt et al., 2006). This has been quantified in elite distance runners as a 20-day exposure to LHTL conditions increased their exercise economy by 3.3% at submaximal pace, without significant increase of haemoglobin mass (Hb_{mass}) (Saunders et al., 2004).

It is now evident that the heterogeneity of training, through periodisation and exposure to different stressors (hypoxia), enables and triggers different neuromuscular adaptive mechanisms. These have been well described in the literature in both strength and endurance training, usually recruiting diverging adaptive molecular mechanisms (IGF pathway vs adenosine monophosphate kinase (AMPK) pathway, respectively) (Coffey & Hawley, 2007; Nader, 2006). Changes in performance variables have also been explored following different training modalities (García-Pallarés et al., 2010). Haematological and

biochemical adaptations, however, have not been as well explored. After concluding that alterations in volume and intensity during a training season produced changes in several haematological parameters, Borges et al. (2012) advised that haematological monitoring is fundamental to the selection of optimum training loads. By closely controlling haematological variations, potential health risks (such as anaemia and increased susceptibility to infection) can be identified before they hinder athletic performance.

2.2) Immune System

2.2.1) Overview

The immune system is a vital host defence system dedicated to the identification and destruction of foreign pathogens or infected/abnormal cells. It does so by evoking several strategies to discriminate self from non-self, such as recognizing molecular patterns in a pathogen's surface or altered/infected host (own) cell (Medzhitov & Janeway, 2002). The immune system may be didactically divided into subsections providing innate and adaptive immunity. Innate immunity grants the initial protection against infections through physical barriers (epithelium), humoral (soluble) components and an array of cellular components, which will be discussed further below. Adaptive immunity consists of an antigen-specific response through lymphocytes - T cells and B cells. One of the most distinct features of the adaptive immunity is the ability to create "memory" towards a specific pathogen, allowing a more vigorous and rapid response if the host ever encounters the same invader again. In contrast to the innate immune response, this is not an immediate response and usually requires days or even weeks to develop (Pathak & Palan, 2012). The adaptive immune system is highly dependent on the antigen presentation and activation provided by innate immune cells, such as dendritic cells (DC) (Banchereau et al., 2000; Male, Brostoff, Roth, & Roitt, 2013). Beutler (2010a) affirms the importance of the innate immune system by suggesting that in a non-sterile environment, survival without it would be impossible.

The cellular components of the innate immune system include haematopoietic cells of myeloid (granulocytes, monocytes/macrophages, mast cells and dendritic cells) and lymphoid (natural killer [NK] cells) origin (Turvey & Broide, 2010). The myeloid progenitors, more specifically granulocytes (mostly neutrophils) and

monocytes/macrophages, are phagocytic cells that recognize, engulf and destroy pathogens (Beutler, 2004). Due to the vital immediate response nature of innate immunity and the predominant concentration of phagocytes amongst all immune cells, this research will focus on this population, more specifically, neutrophils, that make up 60-70% of circulating leucocytes (Amulic, Cazalet, Hayes, Metzler, & Zychlinsky, 2012).

2.2.2) Polymorphonuclear Neutrophils (PMN)

Under optimal physiological conditions, approximately $16x10^{10}$ neutrophils are produced per day (Liu, Wu, Wesselschmidt, Kornaga, & Link, 1996; Moore, Sheridan, Allen, & Dexter, 1979). In response to infection, the concentration of neutrophils in peripheral circulation can rapidly increase up to 10-fold (Lieber et al., 2004; Liu et al., 1996). The ability to rapidly increase neutrophil numbers in the circulation requires that a large pool of neutrophil progenitors be readily available. The largest neutrophil pool is found in the bone marrow, with approximately $18x10^{11}$ neutrophil progenitors in every developmental stage (Lieber et al., 2004).

Neutrophils originate from a pluripotent haematopoietic stem cell, following stimulation by specific growth factors (Gabrilovich, 2005), such as granulocyte colony stimulating factor (G-CSF) (Barreda, Hanington, & Belosevic, 2004; Chatta, Price, Allen, & Dale, 1994; Liu et al., 1996). The initial development is characterized by a mitotic stage lasting approximately 7.5 days. In this stage three distinct phases can be observed: the myeloblast, promyelocyte and myelocyte (Bainton, Ullyot, & Farquhar, 1971). A post mitotic stage, reported to last approximately 6.5 days, then follows where cells differentiate, in turn, into metamyelocytes, band cells and finally segmented (mature) PMN (Bainton et al., 1971).

Cytoplasmic granules are formed throughout PMN maturation in the bone marrow. Three granules have been identified and are classified as azurophilic (primary), specific (secondary) and gelatinase (tertiary) granules. Azurophilic granules appear early in maturation during the promyelocyte stage and are reduced in number by mitosis (Bainton, 1999; Bainton et al., 1971). Specific granules are formed later during the myelocyte stage and continue their development until the band cell phase (Borregaard & Cowland, 1997).

Tertiary granules have been shown to be derived from secondary granules and are formed in the band cell phase (Bainton, 1999; Borregaard, Sørensen, & Theilgaard-Mönch, 2007). Therefore, the mature circulating neutrophil – the segmented neutrophil – contains the three major granule types (Bainton, 1975), as well as secretory vesicles.

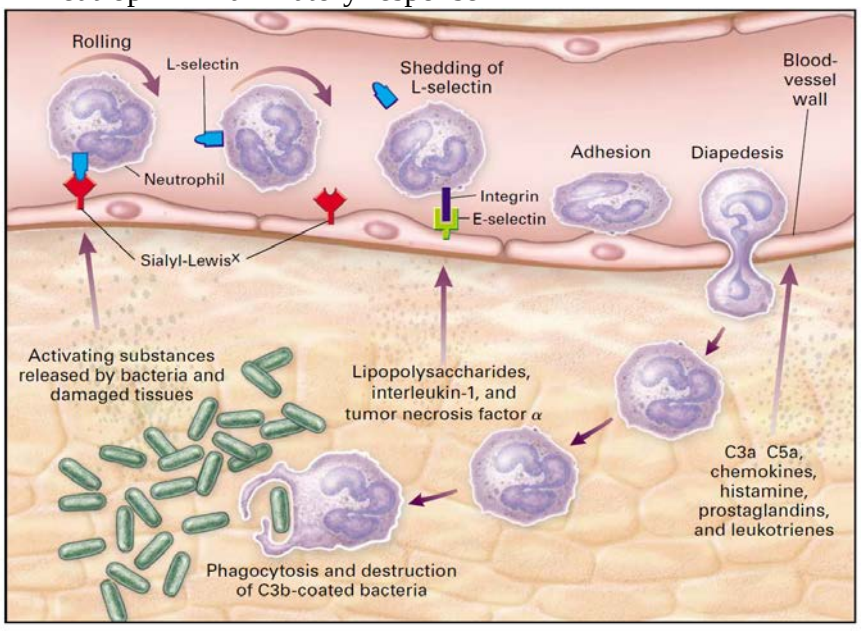
Azurophilic granules contain myeloperoxidase (MPO), proteolytic enzymes (e.g. elastase, cathepsins, proteinase-3), antimicrobial defensins, lysozyme and BPI (Nauseff & Clark, 2010). Granules may be identified by specific molecules on their membrane, termed clusters of differentiation (CD), CD63 and CD68 (Faurschou and Borregaard 2003). Specific granules vary greatly in size and composition and, as previously described, enable formation of tertiary granules (Bainton, 1999; Borregaard et al., 2007). Specific granules express CD11b, CD18, CD66 and cytochrome b_{558} on their membrane and contain in its matrix, lactoferrin (LF), lysozyme and B12 binding proteins (amongst other substances) (Faurschou & Borregaard, 2003). These granules are categorised according to their protein content. It has been reported that specific granules may contain only LF (16%), only gelatinase (24%), or a combination of both LF and gelatinase (60%) (Bainton, 1999). MPO and LF play an important role in the oxidative chemistry of the phagosome and anaerobic microbicidal activity, respectively, which will be explored further in this thesis.

Neutrophils have been suggested to have superior phagocytic ability when compared to the mononuclear phagocytes (Silva & Correia-Neves, 2012). Several authors (Levy, 2004; Segal, 2005) have suggested that neutrophilic microbicidal capacity is greater than that of macrophages, attributing this to the diverse assortment of microbicidal mechanisms and antimicrobial molecules stored in the granules with which they are equipped (Borregaard & Cowland, 1997; Segal, 2005). Nathan and Shiloh (2000) identified a more prominent production of ROS by neutrophils when compared to that of macrophages. Locksley et al. (1987) have ascribed the lower antimicrobial activity in macrophages, when compared to neutrophils, to the loss of MPO as they mature from monocytes (in circulation) to macrophages (in tissue) (Klebanoff, 2005). Also, abundant in neutrophils, but scarce or even lacking in macrophages, are antimicrobial proteins such as defensins and cathelicidins (Ganz, 2003a; Lehrer & Ganz, 2002; Selsted & Ouellette, 2005), LF (Levay & Viljoen, 1995), as well as the bactericidal/permeability-increasing protein (BPI)(Weiss & Olsson, 1987).

2.2.3) Neutrophils and the Inflammatory Response

Circulating mature neutrophils are often the first cells to arrive at an infection site, triggering a local inflammatory response. Early in the inflammatory response, neutrophils are displaced from blood and marginated along the endothelial surface. Stimulated by selectins, rolling neutrophils initiate adhesive interaction with the endothelium (Figure 2-1). Pro-inflammatory mediators, such as tumour necrosis factor α (TNFα) and interleukin (IL) 1-β , induce surface expression of E-selectin, which in turn increases endothelial expression of intercellular adhesion molecule (ICAM)-1 (Muller, 2002). ICAM-1 then binds to β_2 (leucocytes) integrins which are heterodimeric structures that contain α chains (CD11a, CD11b, CD11c, CD11d) and a common β chain (CD18). The binding of CD11b/CD18 complex with the complement activation product C3bi facilitates adhesion and allows diapedesis (Warren, 2010). The neutrophil will then emigrate and move through the interstitium. Specific chemotactic receptors on the neutrophil surface, such as N-formyl-methionyl-leucyl-phenylalanine (fMLP), complement derived peptide C5a, bioactive lipid product platelet activating factor (PAF) and cytokines (e.g. IL-8), are occupied, thus activating the neutrophil and promoting directional movement, known as chemotaxis, towards the pathogen (Cohen, 1994).

Figure 2-1 – Neutrophil inflammatory response



Neutrophils are the first cells recruited to the inflammatory site, mostly via interaction of selectins and integrins on both neutrophil and vascular endothelium. This interaction slows down neutrophilic rolling and promotes firm adhesion. Through conformational changes in the cytoplasm the neutrophil migrates into the tissue. There the interaction between receptors on the neutrophil's surface and chemoattractants, initiates phagocytosis and consequently, extermination of the foreign pathogen. *Reproduced with permission from Delves and Roitt (2000), Copyright Massachusetts Medical Society.*

Neutrophils can identify their targets directly through recognition of pathogen-associated molecular patterns (PAMPs) (such as lipopolysaccharide; LPS) by pattern-recognition receptors (PRR) located in their membrane, or indirectly where a target has been coated by serum opsonins, such as complement (i.e. C3b and iC3b) and immunoglobulin (Ig), particularly IgG (Cohen, 1994). Neutrophils express on their surface receptors that bind to the Fc portion of the IgG molecule, particularly FcγRII (CD32) and FcγRIII (CD16). To recognize complement, neutrophils express complement receptors 1 and 3 (CR1 and CR3) which recognize C3b and iC3b, respectively. Data suggests that calcium-dependent phagocytic pathways are mediated by Fcγ receptors, whereas CR1 and CR3 mediate calcium-independent phagocytic pathways (Nauseff & Clark, 2010). Once occupied, these receptors trigger engulfment and microbicidal activity of the phagocyte. The pathogen is then trapped within a phagosome, where the neutrophils use both O_2-independent and O_2-dependent mechanisms to kill the invader.

The O_2-independent mechanism recruits the contents of the three types of granule to exterminate the pathogen. Stimuli such as the occupation of C5a, PAF and the fMLP membrane receptor trigger the degranulation process (Tintinger, Steel, & Anderson, 2005). Such receptors belong to the 7-transmembrane G-protein-coupled family of receptors (Tintinger et al., 2005). Once engaged, the receptors, controlled by G-protein subunits Gα and Gβγ, activate B isoforms of phospholipase C (PLC) (Tintinger et al., 2005). Active PLC cleaves inositol 4,5-biphosphate (PIP2) into inositol triphosphate (IP3) and diacylglycerol (DAG) (Nelson & Cox, 2008). IP3 then binds to Ca^{2+} mobilizing receptors on the intracellular storage vesicles, releasing Ca^{2+} into the cytosol (Lacy, 2006). The gradual increase in cytosolic Ca^{2+} concentration via the phosphoinositide cascade causes the granule contents to be emptied sequentially (secretory vesicles, tertiary granules, secondary granules and primary granules) either into the phagosome or into extracellular space (Faurschou & Borregaard, 2003). CD66 is a secondary granule marker, detected in low density in the plasma membrane of resting neutrophils (Borregaard & Cowland, 1997). As the degranulation process occurs, CD66 is mobilized to the surface from its intracellular location, the secondary granules (Ducker & Skubitz, 1992). The increase in the surface marker CD66 characterizes the sequential degranulation process. The antimicrobial arsenal provided by the granules includes defensins, BPI, lysozyme, peptidoglycan recognition proteins (PGRP), neutrophil gelatinase-associated lipocalin (NGAL), cathelicidins and LF. These agents act to disrupt anionic bacterial surfaces,

increasing their permeability (Mayer-Scholl, Averhoff, & Zychlinsky, 2004) and eventually degrading bacterial proteins.

The O_2-dependent mechanisms use granule content (particularly from secondary granules) (Lee, Harrison, & Grinstein, 2003) from these PMN leucocytes to create a hostile environment for the pathogen inside the phagosome. In a process of intense O_2 consumption referred to as "respiratory or oxidative burst", electrons are taken from NADPH in the cytoplasm, via the enzyme NADPH oxidase (enzyme commission EC 1.6.3.1) and transferred onto O_2 in the vacuole to produce superoxide anion (O_2^-) (Wientjes & Segal, 1995). Subsequent reactions involving MPO, which will be explored further in this thesis, lead to the formation of other toxic species, including hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), hydroxyl radical ($OH\cdot$) and singlet oxygen ($^1O_2$) (Clark, 1999).

Recently, a novel neutrophilic antimicrobial mechanism has been described (Brinkmann et al., 2004; Fuchs et al., 2007), where neutrophils release extracellular traps (NETs) composed of chromatin and granular proteins. NET formation follows a particular pattern which culminates in the disintegration of the nuclear envelope into the vesicles and the disappearance of granular membranes, allowing the mixing of nuclear, cytoplasm and granular components (Fuchs et al., 2007). Fuchs et al.(2007) describe this process to be dependent on the generation of ROS by NADPH oxidase. This serves to illustrate the importance of ROS formation as it contributes to two antimicrobial pathways: intraphagosomal killing in live neutrophils and NET-mediated killing post mortem (Brinkmann & Zychlinsky, 2007).

Besides their widely reported effector functions, neutrophils are now known to provide a substantial immunomodulatory links between innate and adaptive immunity (reviewed thoroughly in Mantovani et al., 2011 and Jaillon et al. 2013). Synthesis and release of chemokines (CXC and CC) and of cytokines such as IL-1 (α and β), TNFα and IL-6 by neutrophils modulates activity of B and T lymphocytes (Cassatella, 1995; Lloyd & Oppenheim, 1992; Scapini et al., 2000; Tecchio, Micheletti, & Cassatella, 2014). This activation of T-lymphocyte sub-populations (TH1, T_H17 and T_{REG}) in turn promotes release of neutrophil-attracting chemokines (i.e. CXCL8) which increases neutrophil survival and increase expression of CD11b (Mantovani et al., 2011).The recognition of a pathogen through C-type lectin receptors or toll-like receptors trigger (via DAP12 Syk

and myeloid differentiation factor 88 pathways, respectively) the phosphorylation of p38 and phosphatidylinositol 3-kinase (PI3K) and Akt pathways, culminating in the production of IL-10 by murine neutrophils (Cassatella, Locati, & Mantovani, 2009; Zhang, Majlessi, Deriaud, Leclerc, & Lo-Man, 2009). Further cross-talks between neutrophils and DC via CD18 (neutrophils) and DC-SIGN (monocyte-derived DC), promotes maturation of DC (Jaillon et al., 2013), which may result in NK cell activation (IFN-γ production) (Costantini et al., 2010).

2.2.4) The Acute-Phase Response

A coordinated series of systemic and metabolic events including fever, leucocytosis, intracellular iron sequestration and increase of specific hepatic plasma proteins, occurs in response to trauma, stress, inflammation and/or infection, independent of recognition of foreign pathogens. The collection of these non-specific host defence mechanisms, termed the acute phase response (APR), involves not only components of the immune system, but also endocrine and central nervous systems, liver, pancreas, kidneys, and skeletal muscle (Cannon & Blumberg, 2000; Kushner, 1982). Macrophages at the inflammatory site produce cytokines such as IL-1β, TNFα, IFNγ and IL-6, which unleash the systemic APR. As the APR is initiated, there is an increase in the concentration of circulating cytokines such as IL-1β, TNFα, IFNγ and IL-6 (Bode, Albrecht, Haussinger, Heinrich, & Schaper, 2012). These are thought to mediate production of acute-phase proteins (APP) including C-reactive protein, ferritin, proteins of the complement system (i.e. C3), hepcidin, amongst many others reviewed in Gabay & Kushner (1999), Gruys et al. (2005), and Samols et al. (2003).

2.2.5) Exercise and Immune Function

Increased attention is being paid to athletes' immune health and competency. An ill athlete is unable to keep his/her training program, which will, most likely, hinder future performances. The majority of elite athletes experience URTI symptoms at a similar rate to the general population (Papacosta & Nassis, 2011; Walsh et al., 2011), reportedly one to four cases per year (Fricker et al., 2000). Such episodes in elite athletes do not follow the seasonal patterns observed in the general population (Broadbent, 2011), but rather

predominantly occur during or around competition. Many studies (Broadbent, 2011; Gleeson, 2006, 2007; Pedersen & Hoffman-Goetz, 2000; Walsh et al., 2011) have shown that symptoms are dependent on the nature of the sporting activity undertaken. Kayak and swimming athletes usually present with illness during HI training and tapering prior to competition (Fricker, McDonald, Gleeson, & Clancy, 1999; Gleeson & Pyne, 2000), while endurance runners commonly present with symptoms after competition (Nieman et al., 2006). Other than URTI, athletes are also at risk of bactericidal and fungal infections, often attributed to environmental (i.e. water-based sports) or to sharing of sporting apparel. In a recent systematic review of 51 peer-reviewed studies from 1990 to 2012, 27.4% of the articles mentioned *Staphylococcus aureus* skin infection and water-transmitted *Leptospira* was found to be the most common infectious agent with a total of 226 cases described (Grosset-Janin, Nicolas, & Saraux, 2012).

Exercise influences humoral and cellular components of the immune system much alike the APR (Gleeson, 2007; Pedersen & Hoffman-Goetz, 2000), as exercise is perceived as stress by the central nervous system and may enhance or suppress immune function (Dhabhar, 2009). As such, sympathetic nerve fibres increase cardiac output resulting in increased shear stress, which has been postulated to increase leucocyte trafficking as the latter are demarginated from vascular pools. Further, release of catecholamines and cortisol by sympathetic nerve fibre stimulation and neuroendocrine (via hypothalamic–pituitary–adrenal (HPA) axis) stimulation of the adrenal gland, respectively, also contribute to the above mentioned demargination (Walsh et al., 2011). Details of the interrelations between exercise and the endocrine system escape the bounds of this thesis but are of utmost importance and have been described extensively elsewhere (Peake, 2013; St-Pierre & Richard, 2013; Walsh et al., 2011).

Additionally, the increase in circulating levels of immune mediators such as heat-shock proteins, chemokines and cytokines is known to modulate post-exercise immune function. ILs have gained much attention in the sporting field, particularly the increase of pro-inflammatory IL-6. IL-6 is a multifunctional cytokine involved in the regulation of the immune response, APR, haematopoiesis, and inflammation (Akdis et al., 2011). Recently Cox et al. (2010) identified an underlying genetic predisposition to high expression of the IL-6 single nucleotide polymorphism (G-174C) in athletes prone to frequent upper respiratory symptoms (URS) when compared to the healthy group – who reported less than two episodes of URS in a year. Such polymorphism impacts on cytokine production

by increasing IL-6 expression (Fishman et al., 1998; Terry, Loukaci, & Green, 2000). Data from this study (Cox et al., 2010) show that the IL-6 high expression genotype was more frequent in the illness-prone group than in the healthy group (20% v 9%, respectively). Moreover, cytokines, such as IL-1β and TNF-α, and the complement system (via complement opsonisation) can recruit cellular components of the immune system to sites of infection, enhancing the extermination of pathogens (McDonald & Levy, 2013). Fielding et al. (1993) showed positive correlation between accumulated IL-1β and neutrophils in skeletal muscle with Z-band damage in untrained men after a 45min downhill run (16% incline) at 70% HR_{max}.

In relation to cellular immunity, exercise is known to cause an overall increase in circulating leucocytes (Simpson, 2013). However, each leucocyte population responds differently to the exercise stimulus. Significant immunological alterations can be observed even after short periods (1-3 weeks) (Gleeson, 2007) of intense training, where studies have reported significant decreases in lymphocyte concentration (Borges et al., 2012; Horn, Pyne, Hopkins, & Barnes, 2010), while NK cells, monocyte and granulocyte concentrations are found to increase (Fielding et al., 2000; Kakanis et al., 2010). At the completion of training, following a reduced workload, known as the taper phase, several studies have also observed a significant decrease in circulating monocyte concentration (Borges et al., 2012; Morgado et al., 2012). The overall fluctuations in leucocyte concentration in response to exercise are explained mostly by the previously mentioned increase in neutrophils, which make up 60-70% of circulating leucocytes (Smith & Pyne, 1997).

When analysing how exercise impacts immune function, even short training periods (1-3 weeks) demonstrated marked reductions in immune components such as salivary Ig-A, monocyte antigen presentation, the number of T cells producing IFN-γ, lymphocyte proliferation and neutrophil function (Gleeson, 2007; Gleeson & Bishop, 2000; Lancaster et al., 2003; Lancaster et al., 2004). These mononuclear and PMN leucocytes are responsible for detecting and killing pathogens, via either specific responses (e.g. B lymphocytes -antibody production) or non-specific responses (phagocytosis), respectively. While the cumulative effects of athletes undertaking multiple training sessions per day have been demonstrated to significantly increase (p<0.001) neutrophil and lymphocyte (T cells – cytotoxic and helper – and NK cells) concentrations in peripheral blood, they have also been found to exhibit a decrease in the function of these

cells (Ronsen, Pedersen, Øritsland, Bahr, & Kjeldsen-Kragh, 2001). Research examining the phagocytic function of neutrophils has shown conflicting results, where studies have demonstrated it to decrease (Chinda et al., 2003; Gabriel et al., 1995), increase or remain unchanged (Ortega Rincon, 1994; Pyne, 1994). Oxidative burst activity per cell has also been shown to decrease significantly ($p<0.001$) in response to exercise (Chinda et al., 2003; Gabriel et al., 1995) .

The influence of exercise on immune function has been the focus of many studies, often with conflicting results, as previously mentioned. However, there is strong agreement that neutrophil function is decreased, despite observed increases in peripheral blood neutrophil concentration, post-exercise. A major constituent of the non-specific immunity, presenting the first line of defence against foreign pathogens, neutrophils are endowed with a range of microbicidal, bactericidal and virucidal functions, which are critical to host defence. As the body's most distinct phagocyte, neutrophils depend on their full complement of killing capacities – adherence, chemotaxis, phagocytosis and microbial killing - to ensure successful extermination of invaders.

Studies that focus on the granular content of neutrophils have demonstrated fluctuations in the cell's functional response post-exercise. While the oxidative burst function has not been shown to be altered immediately post-exercise, significant decreases in its capacity have been observed 3-6 hours thereafter (Morozov, Pryatkin, Kalinski, & Rogozkin, 2003). Accordingly, chemiluminescence (activation resulting from metabolic oxygenation activity, measured by luminol deoxygenation producing a high quantum yield of photons) per neutrophil was found to be the same immediately post exercise and was sustained for the hour to follow (Lieberman, Sachanandani, & Pinney, 1996). Once again, in the post-exercise period analysed, chemiluminescence intensity per neutrophil was shown to be reduced by 22% and 28%, at the third and sixth hour, respectively (Morozov et al., 2003). Morozov et al. (2003) found a significant correlation ratio ($\eta=0.95$; $p<0.001$) between the intensity of the degranulation process and work capacity. Gray et al. (1993) established the existence of a relationship between intense interval training and granulocyte degranulation, through an increase in the expression of receptors for complement fragment C3bi (CD11b/CR3) and the Fc portion of IgG (CD16) 24 hours post-exercise.

It has been suggested that such degranulation stimulates bone marrow granulopoiesis (Delforge et al., 1985; Metcalf, 1997; Morozov et al., 2003). The decrease in oxidative burst capacity was then attributed to the release of immature bone marrow neutrophils, a phenomenon known as “left shift”. Suzuki et al.(2003) demonstrated that there was a significant increase in band (non-segmented) neutrophils from pre- to post- marathon race, 310 to 3650 cells/μL, respectively. Post-race plasma showed an increase in the concentration of cytokines, particularly IL-6, IL-8, IL-10, G-CSF and monocyte chemotactic protein 1 (Nieman et al., 2005; Suzuki et al., 2003). Recent findings have identified a positive correlation between increased levels of IL-6 and G-CSF and neutrophil mobilization from the bone marrow (Suzuki et al., 2002). It is well established that both IL-6 and G-CSF are increased post-exercise (Peake, 2002; Yamada et al., 2002). Interestingly, IL-6 and G-CSF were also found to facilitate neutrophil degranulation (Borish, Rosenbaum, Albury, & Clark, 1989; Jiang, Puntis, & Hallett, 1994; Suzuki et al., 2000) and consequently are often used as markers for neutrophil activation (Camus et al., 1998; Niess et al., 1999; Suzuki, Totsuka, et al., 1999).

Although the presence of immature neutrophils in the circulation has been discussed by many authors, there is limited literature quantifying the different granulocyte phenotypes according to their maturity post-exercise. Even scarcer are studies in the sporting field that correlate immature neutrophil phenotypes to their functional capacity.

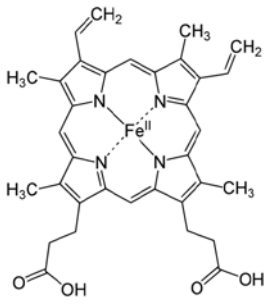
2.3) Iron

2.3.1) Physiological Roles of Iron

Iron is an essential transition metal that possesses numerous biological roles. This micronutrient is required for adequate erythropoietic function, oxidative metabolism, and cellular immune responses. ID, clinically defined as serum ferritin <35μg/L; haemoglobin >115 g/L; Tf saturation >16% (Peeling, Dawson, Goodman, Landers, & Trinder, 2008), is the most common nutrient deficiency in the world. According to the World Health Organization, over 30% of the world’s population is anaemic and about one billion suffer from iron deficiency anaemia (IDA) (Butcher, Chahal, Nayak, Sinclair, Henriquez, Sapey, O’Mahony, et al., 2001).

Iron has diverse biochemical functions in living organisms. These can be divided into (1) structural, (2) electron transport, (3) dioxygen binding and (4) catalytic roles (Crichton, 2001). Iron can be found in iron containing proteins, such as haemoproteins, iron-sulfur (Fe-S) proteins, and mononuclear and dinuclear non-haem iron enzymes, all of which are essential for cellular metabolic functions. The most common catalytic use of iron is when it is incorporated into a prosthetic group called haem. Haem consists of a single iron atom, in its ferrous state (Fe^{2+}), bound to a complex organic structure, protoporphyrin IX (Figure 2-2). Haemoproteins can be further subdivided into O_2 carrying proteins (haemoglobin and myoglobin), activators of molecular O_2 (cytochrome oxidase, peroxidases, catalases and cytochrome P450), and electron transport proteins (cytochromes) (Crichton, 2001). Haemoglobin and myoglobin are reversible O_2 binding proteins that transport and store O_2, respectively, throughout the body. Cytochromes participate in a number of respiratory chains, interacting with other compounds, accepting, and redistributing electrons.

Figure 2-2 – Haem molecule



The chemical properties of iron define its biological importance. Iron can transition between the ferric (Fe^{3+}) and Fe^{2+} states through one-electron oxidation-reduction reactions (Hentze, Muckenthaler, & Andrews, 2004). When not adequately sequestered by proteins, iron can participate in Fenton reactions, where Fe^{2+} reacts with hydrogen peroxide (H_2O_2) or lipid peroxides, generating Fe^{3+}, OH^-, and the highly reactive and toxic hydroxyl radical ($OH\cdot$), or lipid radicals such as lipid alkyl radical ($LO\cdot$) and lipid alkoxyl radical ($LOO\cdot$) (Hentze et al., 2004). These radicals damage cellular membranes, proteins, and DNA. Due to this reactivity, both intra and extra cellular iron is usually bound to proteins. Therefore, iron homeostasis requires tight control of its uptake, storage, and distribution.

2.3.2) Iron Metabolism

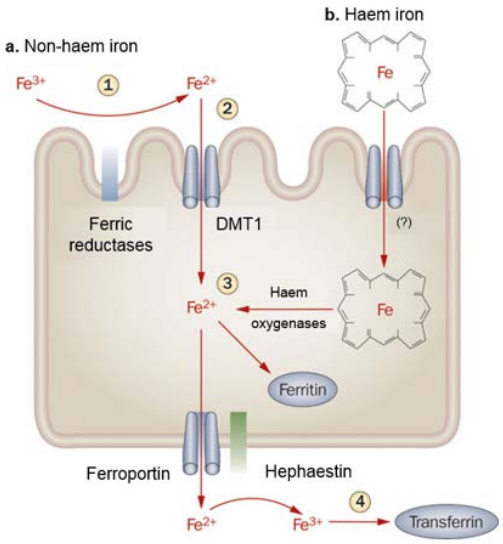
A normal healthy adult contains 3-5g of iron. Perhaps two thirds of this is present in haemoglobin in circulating erythrocytes, with much of the rest distributed between iron-containing proteins in all cells. Approximately 10-20mg of iron in the body is in excess of immediate metabolic requirements and is stored mainly in hepatocytes and reticuloendothelial macrophages. The maintenance of body iron levels is critical for health and the capacity for physical work. Of the 15-20mg of iron present in a normal daily diet, only 1-2mg/day is absorbed (Hentze, Muckenthaler, Galy, & Camaschella, 2010). This is much less than the 20-25mg/day which is required for erythropoiesis. To meet these requirements, there is extensive internal turnover of iron, with iron from senescent erythrocytes being returned to the transferrin-bound iron pool in the plasma by the reticuloendothelial system (RES). The transferrin-bound iron pool turns over 5-7 times per day, serving to meet erythropoiesis requirements in the bone marrow (Andrews, 2008; von Drygalski & Adamson, 2012).

If the internal recycling of iron is insufficient to meet metabolic (predominantly erythropoietic) needs, intestinal iron absorption becomes important. Inorganic (non-haem) dietary iron is absorbed by the duodenal enterocytes after being reduced to Fe^{2+} by brush border ferric reductases. Divalent metal-ion transporter 1 (DMT1) then transports Fe^{2+} across the brush border membrane via a proton-coupled mechanism (von Drygalski & Adamson, 2012; Wang & Pantopoulos, 2011). Once in enterocytes, Fe^{2+} can either be stored within ferritin, or exported via ferroportin across the basolateral membrane and into the circulation (Figure 2-3). The latter process requires an iron oxidase, hephaestin, for maximum efficiency. Haem iron absorption is less well understood. Haem must be released from proteins (e.g. haemoglobin, myoglobin) through proteolytic activity in the lumen of the stomach and duodenum. After movement from the lumen into the enterocyte, by a mechanism that has not yet been defined, iron is released from the protoporphyrin ring by haem oxygenases (Hartmann & Bissell, 1982; Raffin, Woo, Roost, Price, & Schmid, 1974; Tenhunen, Marver, & Schmid, 1968). Haem-derived iron subsequently appears to exit the enterocyte through the same pathway as non-haem iron, i.e. via ferroportin (Anderson, Frazer, McKie, Vulpe, & Smith, 2005).

Iron absorption is influenced by both systemic regulators, such as hepcidin, and at the enterocyte level (Kühn, 2009; Simpson & McKie, 2009). Enterocyte O_2 tension

influences the transcription factor hypoxia-inducible factor 2α (HIF-2α), which in turn regulates the transcription of DMT1 and ferroportin (Peyssonnaux, Nizet, & Johnson, 2008; Recalcati, Minotti, & Cairo, 2010). Von Drygalski and Adamson (2012) showed that when the enterocytes are hypoxic or iron deficient, DMT1 and ferroportin expression is upregulated, thus promoting dietary iron absorption. While enterocyte iron levels can influence HIF-2α activity, they can also influence the activity of iron regulatory proteins 1 and 2 (IPR1/ IRP2) (Galy, Ferring-Appel, Kaden, Gröne, & Hentze, 2008). Both IRP1 and IRP2 bind to iron responsive elements (IREs) in the untranslated regions of certain target mRNAs when iron is scarce. Such binding stabilizes transferrin receptor 1 (TfR1) and DMT1 messenger RNA (mRNA), and decreases the translation of the mRNA for ferritin, ferroportin and HIF-2α (Sanchez, Galy, Muckenthaler, & Hentze, 2007).

Figure 2-3 – Absorption mechanism of haem and non-haem iron by enterocytes



a. Non-haem iron absorption. (1) Non-haem iron is reduced from Fe^{3+} to Fe^{2+} either chemically (by gastric acid) or through the action of brush border reductases. (2) Fe^{2+} is taken up into mucosal cells via DMT1. (3) Once inside the intestinal epithelial cell, iron either binds to ferritin or is transported via ferroportin into the circulation. Hephaestin oxidizes Fe^{2+} to Fe^{3+}. (4) Fe^{3+} binds to transferrin. **b.** Haem iron absorption. Haem is transported across the brush border membrane via mechanisms that are still poorly understood. Within the enterocyte, iron is liberated from its porphyrin framework by haem oxygenases and enters the same pool as non-haem iron. *Re-printed and adapted, with permission, from Stein, Hartmann, and Dignass (2010)*

Recently, many studies have highlighted hepcidin as the key regulator of systemic iron homeostasis, affecting both iron absorption and the recycling of erythrocyte-derived iron by macrophages (Collins, Wessling-Resnick, & Knutson, 2008; De Domenico, Ward, & Kaplan, 2007; Ganz, 2003b; Nemeth & Ganz, 2009; Nicolas et al., 2002; Young et al.,

2009). Hepcidin is a 25-amino acid peptide hormone produced primarily by hepatocytes and released into the circulation (Krause et al., 2000; Park et al., 2001). Regulation of hepcidin production is largely transcriptional, and mRNA levels increase in response to iron loading (via bone morphogenic protein (BMP) / sons of mothers against decapentaplegic homologue protein (SMAD) signalling pathways) and inflammatory stimuli (i.e. cytokines, namely IL-6) (Andriopoulos Jr et al., 2009; Camaschella & Silvestri, 2008; De Domenico et al., 2007; Ganz, 2011). In contrast, transcription rates are decreased under conditions of hypoxia and iron withdrawal/deficiency, as the actions of HIF-1 are also known to downregulate hepcidin expression (Beutler, 2010b).

Hepcidin acts by binding to ferroportin on the cell surface and facilitating its internalization and degradation (Collins et al., 2008). This leads to iron sequestration inside the cell. Consistent with this, mice injected with hepcidin showed an 80% drop in serum iron levels within one hour (Rivera et al., 2005). The response of hepcidin to body iron levels is proportional to the level of transferrin saturation (Collins et al., 2008). The effects of hepcidin on iron status have been demonstrated in many studies (Auersperger et al., 2012; Ganz, 2006, 2011; Nemeth & Ganz, 2009; Nicolas et al., 2002; Roe, Collings, Dainty, Swinkels, & Fairweather-Tait, 2009; Viatte & Vaulont, 2009; Zimmermann et al., 2009).

2.3.3) Iron Deficiency Anaemia

Iron deficiency anaemia (IDA) is characterized by a staged decrease in iron levels. Initially a negative iron balance may be observed, followed by iron depletion. In these stages, the anaemia may produce normocytic and normochromic erythrocytes, and may not have overt symptoms. Further depletion of iron stores leads to iron-deficient erythropoiesis and consequently IDA. The rapid progression and lack of pronounced symptoms makes monitoring crucial to ensure that iron depletion does not escalate into IDA. It is only at the iron-deficient erythropoiesis stage that symptoms will arise. These later stages are characterized by microcytic and hypochromic erythrocytes, with decreased erythropoiesis in the bone marrow resulting in low haemoglobin levels (Clark, 2008). In IDA, serum ferritin is decreased, and there are increases in soluble transferrin receptor and transferrin concentration (measured as total iron-binding capacity) (Table 2-3) (Beutler, 2010b).

Table 2-3 – Sequential changes in iron status

Measure	Normal	Early Negative Iron Balance	Iron Depletion	Iron-Deficient Erythropoiesis	IDA
Bone marrow iron (*)	2-3*	1*	0-1*	0	0
TIBC (µg/dL)	330±30	330-360	360	390	410
Ferritin (µg/L)	100±60	<25	20	10	<10
Iron Absorption (%)	5-10	10-15	10-15	10-20	10-20
Plasma Iron (µg/dL)	115±50	<120	115	<60	<40
TSAT (%)	35±15	30	30	<15	<15
Erythrocyte protoporphyrin (µg/dL)	30	30	30	100	200
Erythrocytes	Normal	Normal	Normal	Normal	Microcytic + Hypochromic
sTfR	Normal	Normal-High	High	Very High	Very High

TIBC = total iron-binding capacity. TSAT = transferrin saturation. sTfR=soluble transferrin receptor * Represents estimates of iron stored in bone marrow through a 6 -point scale: 0=iron absent 1=iron decreased 2-3=normal 4=iron increased 5= iron massively increased. *Adapted from Clark (2008).*

2.3.4) The Anaemia of Inflammation

Also known as the anaemia of chronic disease (ACD), this anaemia is characterized by a low serum iron level, a low to normal transferrin level, normal serum transferrin receptor and a high to normal ferritin level (Beutler, 2010b).

Table 2-4 – Comparison of laboratory measures in IDA and ACD

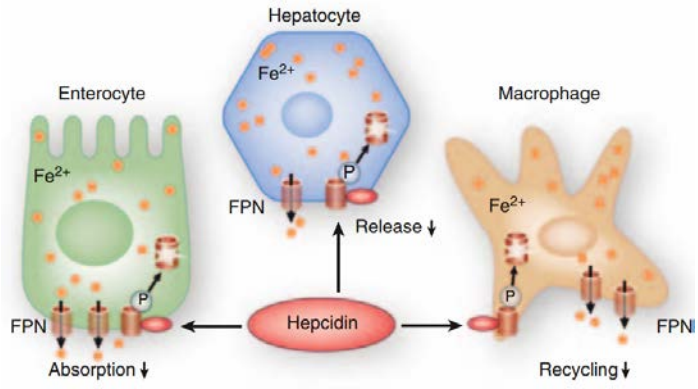
Laboratory Measures	IDA	ACD
Serum Ferritin	Reduced	Normal to Increased
Serum Iron	Reduced	Reduced
Transferrin	Increased	Reduced to Normal
Transferrin Saturation	Reduced	Reduced
Mean Corpuscular Volume	Reduced	Reduced to Normal
sTfR	Increased	Normal
Haemoglobin	Reduced	Reduced
sTfR/log ferritin ratio	High (>2)	Low (<1)
Cytokine Levels	Normal	Increased

sTfR= Soluble transferrin receptor. *Adapted from Clark* (2008)

The ACD is accompanied by an increase of cytokine levels (Clark, 2008) (Table 2-4). Of particular interest are the increases in TFN-α, IL-1, IL-6 and interferon-γ (IFN-γ) (Weiss & Goodnough, 2005). TNFα and IL-1 induce the synthesis of ferritin (the major iron storage protein) by macrophages and hepatocytes, thus facilitating increased iron storage within the RES system (Weiss, 2005). IL-6 triggers the synthesis of hepcidin by hepatocytes and its release into the circulation. Hepcidin binds to ferroportin, the iron exporter on the surface of most body cells, and the complex is then internalised and

degraded, locking iron within the cell resulting in a decrease in serum iron (Collins et al., 2008; De Domenico et al., 2007; Weiss & Goodnough, 2005) (Figure 2-4). IFN-γ also contributes to the inhibition of iron delivery to the plasma as it stimulates DMT1 synthesis and down-regulates ferroportin expression (Weiss & Goodnough, 2005).

Figure 2-4 – The mechanism of iron withholding in the ACD



Reprinted with permission Cui, Wu, and Zhou (2009).

2.3.5) Iron and Immune Function

Optimal performance is also dependent on the health status of the athlete. A less explored avenue in which iron possibly influences performance is via alterations in immune function. The well-known inflammatory response to exercise has provided new perspectives on the decreased iron levels often seen in athletes. The role of inflammatory products such as cytokines and hormones is becoming more evident in iron metabolism. The inflammatory cytokine IL-6, known to be increased post exercise, is intimately related to the up-regulation of hepcidin activity (Northoff & Berg, 1991; Peeling et al., 2009a; Roecker, Meier-Buttermilch, Brechtel, Nemeth, & Ganz, 2005). As previously described, hepcidin is a key regulator of iron metabolism. Studies have shown that IL-6 is not only released as a product of exercise induced inflammation but also as a product of muscle contraction (Febbraio & Pedersen, 2005; Helge et al., 2011; Keller et al., 2001; Steensberg et al., 2002). Most of the IL-6 present in circulation post-exercise is muscle derived, and its levels reflect exercise intensity and duration (MacDonald, Wojtaszewski, Pedersen, Kiens, & Richter, 2003; Pedersen, 2011).

Not only is iron fundamental to normal cell differentiation and proliferation, it is an essential structural and functional component of peroxidise-generating and nitrous oxide

generating enzymes (Beard, 2001). Such enzymes are responsible for effective microbial killing, hence the importance of maintaining normal iron stores. Iron is also a regulatory factor in cytokine production and action as well as in the development of cell mediated immunity (Kumar & Choudhry, 2010).

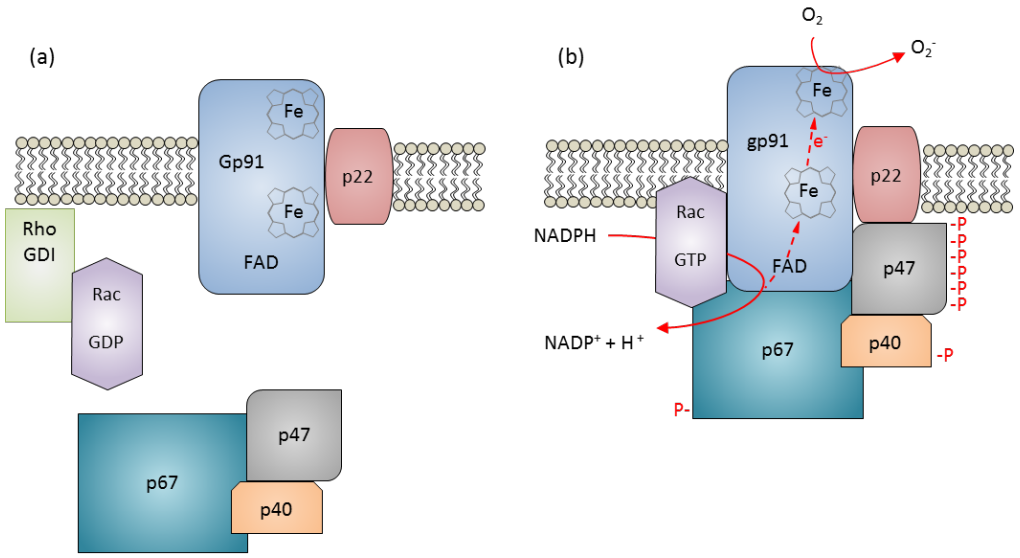
The effects of ID on erythropoiesis are well known (Cavill, 2002). However, this is not the case for myelopoiesis, even though iron is an essential component of myeloid progenitor cells, particularly granulocytes. IDA may cause a hyper-segmentation of neutrophils, as Westerman et al. (1999) observed in 62% of the anaemic adults analysed, compared to 4% of non-anaemic controls. Sipahi et al., (2002) reported that 81% in anaemic children had hypersegmented neutrophils compared to 9% of non-anaemic controls, and this has been demonstrated in other studies (Duzgun, Yildirmak, & Cetinkaya, 2005). Although the clinical significance of hyper-segmentation of the neutrophilic nucleus is still not fully elucidated, Beard and Weintraub (1969) have shown it to be strongly correlated with iron levels, as lobe average in the observed population returned to normal following iron therapy.

The importance of iron for the immune system is most clearly shown during ID (Cherayil, 2010, 2011). Each particular leucocyte population utilises iron in a different manner. Iron plays a key role in lymphocyte proliferation (e.g. DNA synthesis; Seligman et al. 1992), and decreases in iron levels impair such events, particularly in T-cells (Lauffer, 1992). The activity of TH1 helper cells, a sub-population of T cells, is decreased in ID, as manifested clinically by decreased skin-test responses (Lauffer, 1992). Further, lymphocytes and dendritic cells require iron for activation, during which there is increased uptake of iron through the transferrin-transferrin receptor (CD71) system (Brock & Mainou-Fowler, 1983). Although its role is still not fully elucidated, the participation of iron in myelopoiesis is essential to immune competency. It is known that granulocytes carry in their granules essential microbicidal iron-containing proteins such as MPO, iron-binding protein LF, and components of iron-containing enzyme NADPH oxidase.

The NADPH oxidase enzyme complex catalyses the production of O_2^- in phagocytic cells. It is composed of a membrane bound flavohaemoprotein (cytochrome b_{558}), three cytosolic components ($p67^{phox}$, $p47^{phox}$ and $p40^{phox}$) and a low molecular weight G protein (RAC 2 or RAC1) (Babior, 2004; Sheppard et al., 2005) (Figure 2-5). Iron is a component of cytochrome b558, and is present in the two haem prosthetic groups, both of which are

responsible for the assembly of this heterodimer. One haem group selectively binds gp91^{phox}, while the other binds gp91^{phox} and p22^{phox}. These components, combined with a flavin adenine dinucleotide (FAD) make up the NADPH oxidase (Babior, 1999). The FAD and the two haem groups function as a redox pathway, enabling electron transfer across the membrane (Sheppard et al., 2005). Rotrosen et al. (1992) concluded that cytochrome b_{558} is the only obligate electron transporting component of the NADPH oxidase complex, highlighting its role in immune function. In resting neutrophils these cytochrome b_{558} components are distributed between the cytosol and intracellular membranes. The amount of the cytochrome b_{558} complex found in specific granules is four times greater than in the plasma membrane (Vaissiere, Le Cabec, & Maridonneau-Parini, 1999).

Figure 2-5 – NADPH Oxidase



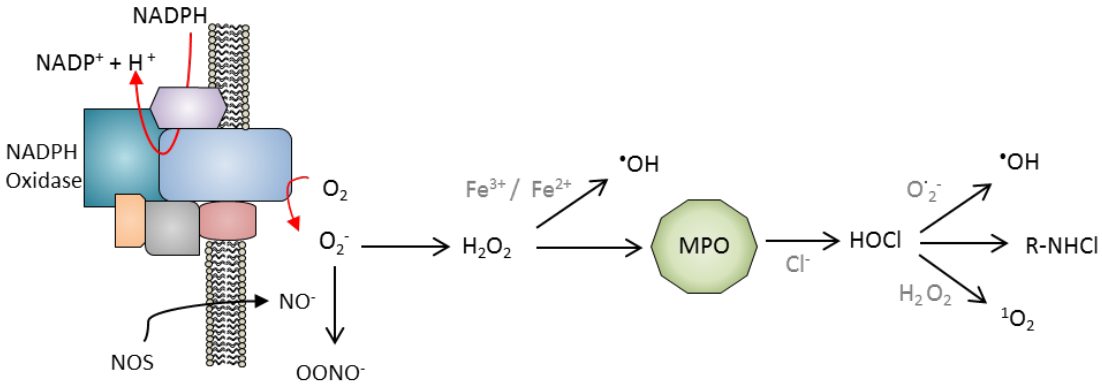
Phagocyte NADPH oxidase activation (a) resting state (b) assembly of components upon activation. *Adapted from Kuijpers and Lutter (2012)*

Vaissiere et al. (1999) propose that, upon activation, specific granules act as the site for functional assembly of the NADPH oxidase complex, with subsequent transfer to the plasma membrane during granule exocytosis. In support of this, Kobayashi et al. (1998), using electron microscopy, identified vesicles and granules as the principal sites of O_2^- production. Any defects in the protein subunits or in the assembly of cytochrome b_{558} results in decreased oxidase activity (Newburger et al., 1983; Vaissiere et al., 1999; Yu, Quinn, Cross, & Dinauer, 1998). Recently, Kurtoglu et al. (2003) demonstrated a

significant decrease in NADPH oxidase activity in subjects with IDA, relative to healthy controls.

More substantial evidence of the importance of the NADPH oxidase system is provided through studies examining the congenital condition of chronic granulomatous disease (CGD), where genetic mutations in any of the genes that encode the NADPH oxidase components described above, the enzyme's activity is reduced or abrogated (Heyworth, Cross, & Curnutte, 2003; Holland, 2010). Consequently, CGD patents are highly susceptible to recurrent infections and prolonged inflammatory reactions (Kuijpers & Lutter, 2012). NADPH oxidase is vital in the generation of superoxide derivatives. These ROS play a key role in pathogen extermination via the oxidative burst. The primary products of NADPH oxidase activity are O_2^- and H_2O_2. O_2^- does not kill bacteria directly and H_2O_2 is only bactericidal at high concentrations, necessitating the formation of secondary oxidants, mostly through the action of MPO (Hampton, Kettle, & Winterbourn, 1998) (Figure 2-6).

Figure 2-6 – Reactions of NADPH oxidase and myeloperoxidase



Through the action of myeloperoxidase hydrogen peroxide forms hypochlorous acid and oxygen and nitrogen reactive species. *Adapted from Hampton (1998).*

MPO is a haem containing enzyme involved, alongside NADPH oxidase, in the formation of ROS and oxidation of biological material (Arnhold, 2004). Utilising products of the oxidative burst, MPO will generate highly anti-microbial ROS, contributing significantly to the neutrophil killing process of microbes (Hampton et al., 1998). MPO contains two identical haems, joined with the apoprotein by two ester linkages and one sulfonium ion linkage (Fiedler, Davey, & Fenna, 2000). Halogenation and peroxidise cycles transform the native enzyme into two distinct forms. The activated forms are able to oxidise

different substrates. Of greater significance is the oxidation of chloride (Cl^-) to hypochlorous acid (HOCl) done exclusively, in mammals, by MPO (Gaut et al., 2001). HOCl reacts with various compounds to form further ROS and nitrogen reactive species (NRS). Many species of bacteria are killed readily by the myeloperoxidase/hydrogen peroxide/chloride system, and HOCl is the most bactericidal oxidant known to be produced by the neutrophil (Hampton et al., 1998). Not surprisingly, neutrophils carry three times more MPO than monocytes (Arnhold, 2004), indicating the importance of this enzyme for effective microbicidal neutrophil function.

ID has been shown to reduce MPO activity (Beard, 2001; Spear & Sherman, 1992). Murakawa et al. (1987) induced ID in rats and demonstrated that MPO activity was decreased by 53%, when compared to a control group. In the same study, MPO activity did not return to baseline values until intramuscular iron supplementation had been provided for seven days.

A member of the transferrin family, the 80kDa iron-chelating protein LF contains two iron-binding sites and is found in secondary granules of mature neutrophils. LF exists in two forms: an iron-free form, apo-lactoferrin, and in an iron-loaded form as holo-lactoferrin. The loading of iron to this protein causes conformational changes to the tertiary structure of LF (Baker & Baker, 2009). In this thesis, wherever LF is mentioned please regard it as apo-lactoferrin unless stated otherwise. Many physiological functions have been attributed to LF, such as cellular growth and differentiation, host defence against microbial infection and inflammation and regulation of myelopoiesis (Legrand & Mazurier, 2010; Lonnerdal & Iyer, 1995). LF has a crucial role in host immunity by not only sequestering iron, due to its high iron affinity, and destabilizing membranes of microorganisms thus limiting their proliferation and adhesion to the epithelial surface, but also, by modulating the overall immune response (Puddu, Valenti, & Gessani, 2009). LF plays a role in the degranulation process by modifying the physiochemical properties of the neutrophil surface (Boxer, Coates, et al., 1982; Boxer, Haak, et al., 1982; Faurschou & Borregaard, 2003). LF has also been described as essential for regulating hydroxyl radical production (Cohen, Britigan, Hassett, & Rosen, 1988), for the regulation of granulopoiesis (Broxmeyer, Smithyman, Eger, Meyers, & de Sousa, 1978), and for the modulation of complement function (Kijlstra & Jeurissen, 1982). Ward et al. (2008) demonstrated reduction in stimulated oxidative burst capacity of neutrophils isolated from LF knockout mice. LF has the ability to control antigen presenting cells and influence

cytokine production by binding to LPS and its receptor (CD14) blocking further activation of pro-inflammatory pathways and tissue damage (Puddu et al., 2009; Ward, Paz, & Conneely, 2005).

In ID, decreased levels of LF may result in decreased phagocytic activity (Bethell & Huang, 2004; Ekiz, Agaoglu, Karakas, Gurel, & Yalcin, 2005) and microbial killing, as iron-lactoferrin may provide the iron needed to catalyse the production of free radicals within phagolysosomes (Lima & Kierszenbaum, 1987). The role of LF in effective antimicrobial activity is supported by findings that patients lacking specific LF containing-granules suffer from recurrent infections (Boxer, Coates, et al., 1982; Breton-Gorius, Mason, Buriot, Vilde, & Griscelli, 1980; Sanchez, Calvo, & Brock, 1992). De Vet and ten Hoopen (1978) found low concentrations of LF in neutrophils in IDA and even lower concentrations in acute inflammation. Such low concentrations could prevent neutrophils from effectively killing engulfed micro-organisms.

2.3.6) Iron and Exercise

Once thought to merely be the product of fluidic shift post-exercise, "sports anaemia" has, after extensive research, generated new scientific reviews and explanations. Nowadays it is known that sports anaemia may be ascribed, in part, to exercise-related events including haemolysis, premature senescence of erythrocytes, depressed Fe^{3+} absorption and increased iron loss via sweat and gastrointestinal tract bleeding (Smith & Roberts, 1994). As previously mentioned, ID is very common in the general population and is frequent among athletes (Koehler et al., 2012). For example, Fogelholm et al. (1995) found that mean iron depletion (measured by serum ferritin) was 14% greater in athletes than in sedentary controls.

Avenues for iron loss due to exercise include sweating, gastrointestinal bleeding, haematuria, haemolysis and the inflammatory response. It is known that iron stores in women are lower than in men – approximately 12% of total body iron in women compared to 30% in men (Gleason & Scrimshaw, 2007). This is a concern for female athletes, who, in many studies, even fail to meet recommended dietary allowances for iron intake, despite having increased dietary iron density (ratio between iron intake and energy intake) when compared to male athletes (Koehler et al., 2012). Koehler et al. (2012)

identified iron depletion (serum ferritin <35µg/L) in 57% of the 97 female elite junior athletes studied. Additionally, female athletes lose significant amounts of iron during menstrual periods, when iron status has been intimately correlated with intensity and duration of the menses (Malczewska et al., 2000).

ID is known to severely affect skeletal muscle, impairing its capacity for oxidative metabolism (Dallman, 1986). Due to the large mass of muscle in the body, several authors (Davies et al., 1984; Davies, Maguire, Brooks, Dallman, & Packer, 1982; McLane et al., 1981) have discussed the impact of this decrease in oxidative metabolic capacity on energy metabolism. Most of the mitochondrial enzymes involved in the oxidative production of ATP contain iron. As haem, iron is present in cytochromes a, b and c, and, as part of Fe-S complexes, iron can be found in NADH dehydrogenase (Complex I) and succinate dehydrogenase (Complex II) (Crichton, 2001; Dallman, 1986). ID affects the iron-containing enzymes in mitochondria according to their location in the ETC (Dallman, 1986). As part of the first reaction in the ETC, Fe-S-containing dehydrogenases are the most severely depleted, followed by cytochrome b and c - Complex III - located in the middle of the ETC (Dallman, 1986; McKay, Higuchi, Winder, Fell, & Brown, 1983). The least affected are cytochromes a and a_3, essential components of Complex IV, which comprise the last reaction of the ETC (Dallman, 1986). Muscle mitochondria content is directly correlated with exercise capacity, notably in endurance exercise (Holloszy & Coyle, 1984). The increase in muscle mitochondrial content and respiratory capacity of the muscle fibres are adaptations induced by endurance exercise (Holloszy, 1967). There is a two-fold increase in succinate and NADH dehydrogenase, NADH-cytochrome c reductases and cytochrome oxidase per gram muscle in rats following a two hours/day running program (Holloszy, 1967), and the total protein content of the mitochondrial fraction increased approximately 60% compared to sedentary rats (Holloszy, 1967), findings later confirmed in human studies (Hoppeler, Lüthi, Claassen, Weibel, & Howald, 1973; Morgan, Cobb, Short, Ross, & Gunn, 1971). However, Perkkio et al. (1985) demonstrated that even though training improved the oxidative capacity in iron deficient rats, it only matched the oxidative capacity of the sedentary rat fed an iron-sufficient diet, failing to match the oxidative capacity of trained rats with normal iron status. Davies et al. (1984) proposed that in ID, the observed defects in $\dot{V}O_{2max}$ were a result of diminished O_2 delivery, whereas decreased endurance capacity was reflective of an impaired muscle mitochondrial function.

Other studies investigating physical performance have shown that ID impairs favourable adaptation to aerobic exercise (Brownlie, Utermohlen, Hinton, & Haas, 2004; Hinton & Sinclair, 2007) and that changes in serum ferritin are positively correlated with changes in ventilatory threshold (Hinton & Sinclair, 2007). DellaValle and Haas (2011) identified a significant relationship between the iron status of non-anaemic female rowers and 2 km time trial results showing that rowers with serum ferritin <20µg/L were 21 seconds slower than rowers with normal iron status. These authors also found that even using a higher serum ferritin cut-off (<25µg/L), iron deficient rowers were still significantly slower (-17.4 seconds p=0.01) than controls (Dellavalle & Haas, 2011). Reductions in aerobic capacity ($\dot{V}O_{2max}$ reduced by 30%) and energy efficiency (measured by slope of the regression of $\dot{V}O_2$ on work output at different work levels on a cycle ergometer - 5% reduction) (Li et al., 1994) have been shown to be proportionate to decline in iron stores (serum ferritin < 30µg/L) and usually corrected with iron supplementation (Haas & Brownlie, 2001; Zhu & Haas, 1998). Dallman (1982, 1986) has established the concept that anaemia limits O_2 distribution to the exercising muscle, whereas tissue ID limits the capacity of the individual to perform oxidative metabolic processes.

As discussed above, iron has been widely associated with athletic performance. It is known that decreased iron status results in decreased levels of haemoglobin and myoglobin production, impairing O_2 carrying capacity and consequently decreasing O_2 distribution to muscles (Beard & Tobin, 2000). Additionally, such alterations in O_2 distribution result in changes to lactate metabolism, leading to increased lactate production and decreased blood lactate clearance (Gregg, Mazzeo, Budinger, & Brooks, 1985). Increased lactate levels have been associated with fatigue and an eventual decrease in the ability to sustain a workload (Diafas, Chrysikopoulos, Diamanti, & Kaloupsis, 2009).

Karl et al.(2010), in a randomized, double-blind, placebo-controlled study with 142 female soldiers, found that iron supplements attenuated decrements in iron status in iron-deficient anaemic subjects, but not in iron deficient or normal subjects. Such a finding is consistent with the rationale that iron is not lost post-exercise, but rather it may be trapped inside the reticuloendothelial system, via the actions of hepcidin on ferroportin. This mechanism also reduces intestinal iron absorption and there is an inverse relationship between circulating hepcidin concentrations and iron absorption in humans (Roe et al., 2009; Young et al., 2009; Zimmermann et al., 2009).

ID, accompanied by hypochromic erythrocytes and haemoglobin was more prevalent in mixed sports such as rowing, when compared to solely aerobic or anaerobic disciplines (Milic, Martinovic, Dopsaj, & Dopsaj, 2011). This group highlighted the increased risk of depletion of iron stores for female athletes in mixed sports. Alaunyte, Stojceska, and Plunkett (2015) suggested that such findings could be attributed to the increase in iron requirement by muscle tissue as an adaptation to the increase in oxygen demands for aerobic metabolism in mixed sports. As it may be expected from its acute phase proteic nature, serum ferritin levels are increased post-exercise. Therefore, Milic et al. (2011) suggested that in intense training periods iron parameters such as serum iron and sTfR should be closely monitored. Further, authors agree that acute effects of exercise, seasonal adaptations, and specific training regimes (i.e. strength or endurance training) may induce changes in haematological parameters (Banfi & Del Fabbro, 2006; Di Santolo et al., 2008; Dopsaj, Sumarac, Novakovic, & Dopsaj, 2008; Milic et al., 2011; Schumacher, Schmid, Grathwohl, Bultermann, & Berg, 2002).

Endurance exercise has been shown to increase transferrin concentrations in plasma by 12% post-exercise, and these remain elevated above pre-exercise levels for seven days (Liesen, Dufaux, & Hollmann, 1977). In a case study of a kayak ultra-marathonist, Rodrigues dos Santos et al. (2007) showed biological and body composition modifications at four time points (12 hours, 3 days, 6 days, and 10 days) after HI exercise (1000km in 17 days, varying from 55 to 85 km per day). The subject's plasma iron concentration before the race was 27.4µmol/L. Levels post-exercise were 17.9µmol/L, 18.6µmol/L, 17.7µmol/L and 22.9µmol/L at 12 hours, 3 days, 6 days, and 10 days, respectively. This shows that the plasma iron concentration was reduced by 35% relative to baseline 12 hours' post-exercise and remained below baseline values, even after 10 days. The slow rate at which iron levels return to baseline post-exercise raises the question of whether this reduced amount of available iron may influence biological processes such as the ETC and erythropoiesis, processes that are known to require iron. Additionally, ID may affect performance by decreasing athletes' immunological competence.

2.4) Athletic Monitoring

2.4.1) The Female Elite Athlete

With increasing number of women participating in sport, physiology exclusive to women must be acknowledged. The uniqueness of the female sex hormones in regulating temperature, substrate utilisation, muscle fatigue and endocrine responses to exercise govern, at least in part, the physiological adaptations in female athletes.

It would be an oversight to undertake a study involving female subjects without alluding to female-specific physiology. While the author appreciates that menstrual cycle and the use of oral contraceptive (OC) are occurring events when working with female athletes, detailed analysis the effects of the menstrual cycle and OC use on exercise performance are beyond the scope of this thesis. The sections below are by no means all-encompassing. For further details on the topic please refer to the in-depth reviews by de Jonge (2003) and Allaway et al. (2016).

2.4.1.1) Menstrual Cycle

With focus on female sex hormones, estrogen and progesterone, the menstrual cycle is divided into 3 phases (1) early follicular phase (low estrogen and progesterone); (2) late-follicular phase (high estrogen and low progesterone); and (3) mid-luteal phase (high estrogen and progesterone) (de Jonge 2003). Contrary to popular belief, most research fails to consistently support the notion that the menstrual cycle phase affects intense (anaerobic/aerobic) performance indicators particularly post-exercise blood lactate concentration, plasma volume, haemoglobin concentration, heart rate, ventilation, and other determinants of $\dot{V}O_{2max}$. Some have suggested that during the mid-luteal phase there is increased lipid metabolism (Hackney 1999, Dombovy 1987), while others have found no significant differences in substrate utilisation during this phase (Niklas et al. 1989, de Souza et al. 1990). For prolonged events, the increased body temperature brought about during the mid-luteal phase may potentially increase cardiovascular strain, decreasing time to exhaustion (de Jonge 2003). Another focus of research has been strength and fatigability where, again, there has been no indication of correlation between the menstrual cycle phase and muscle contractile characteristics (de Jonge et al. 2001).

Nonetheless, OC pills are often prescribed to athletes for cycle regulation (decrease/control athletic amenorrhea), prevent bone density loss (in conditions of estrogen deficiency) and to decrease pre-menstrual symptoms such as water retention, fatigue and irritability (Bennell, White, & Crossley, 1999; Carlson, Curtis, & Halpern-Felsher, 2007). Combined oral contraceptives (COCs) have an estrogen and a progestogen component. Recent formulations available contain a lower dosage of synthetic estrogen, ethinyl-estradiol (EE), hormone thought to spare glycogen utilization whilst increasing lipid utilisation during exercise (Ruby et al., 1997). . Recent attention has been paid to oral contraceptive usage amongst athletes and its implication, if any, in performance and adaptation. The few studies employing female athletes (rather than sedentary or moderately active women) did not show any significant change in performance (Rickenlund et al., 2004; Vaiksaar et al., 2011). Rickenlund et al. (2004) analysed 26 endurance athletes (13 with oligo/amenorrhea and 13 menstruating regularly) and 12 controls for 10 months of treatment with low-dose, monophasic COC (30g EE and 150g levonorgestrel) and found significant increase in body mass (~2.4kg) only in the group with irregular or absence of menses.

In addition to their effects on metabolic responses, oestrogen and progesterone influence the immune system. The local alterations in the ovaries and endometrium during the ovulation and menstruation, respectively, mimics key events in inflammation including tissue oedema, recruitment of immune cells and increase in local production of cytokines and chemokines in endometrial inflammatory mediators such as IL-8 and NF-κB (Critchley, Kelly, Brenner, & Baird, 2001; Hutchinson, Rajagopal, Sales, & Jabbour, 2011; Maybin & Critchley, 2015; Rae & Hillier, 2005). Further, recruitment of NK cells, macrophages, eosinophils, lymphocytes and mast cells during the secretory phase of the menstrual have been attributed to the decline in levels of estrogen and progesterone. Estrogen and progesterone have been shown to affect innate monocytic, NK cells, and granulocytic function (Bouman, Heineman, & Faas, 2005). Miyagi et al. (1992) showed that progesterone increases chemotactic activity of neutrophils while estrogen decreases it. Studies on *in vitro* ROS production by neutrophils incubated with estrogen or progesterone has produced conflicting results (Békési et al., 2000; Cassidy, 2003; Molloy et al., 2003). Further, nitric oxide (NO) production via NO-synthase *in vivo* has been shown to increase with higher levels of estrogen (García-Durán et al., 1999). For a more

in-depth review of the influence of sex hormones in immune function the reader is directed to Bouman et al. (2005) and Muñoz-Cruz (2011).

The expression of cytokines (i.e. IL-4, IL-6) and leucocyte concentration in peripheral blood of regular menstruating women has been shown to vary according to menstrual phase (Angstwurm, Gärtner, & Ziegler-Heitbrock, 1997; Faas et al., 2000). Female sex hormones oestradiol and progesterone have been shown to influence levels of IL-6 *in vitro* and *in vivo* (Angstwurm et al., 1997; Konecna et al., 2000). Exercise-focused research on the influence of OC pills in IL-6 levels have shown that the use of OC pills does not influence the exercise-induced rise in IL-6 (Sim et al., 2015). Further, Sim et al. (2015) showed that post-exercise levels of hepcidin were not affected by OC pills. This same group suggested, after testing subjects in both hormone–depleted and –replenished stages, that the different oral contraceptive cycle phases should not be considered as determinant variables in future studies investigating IL-6 and hepcidin involving monophasic OC pills users.

Comparable to the changes induced the menstrual cycle described above, variations in training protocols induce/ impose distinct effects on not only performance parameters, but also immune and iron homeostasis. Every adaptation detailed above can take place only due to communication between environment and cell (i.e. ligands and receptors). The modulation of training, either by increasing load or volume, is perceived as a stressor. As such, increases in blood flow cause mechanical disturbance to the environment (i.e. blood) and the previously described APR causes a chemical alteration (i.e. increases in APP) to the environment that leucocytes and plasma proteins are in. This inflammatory response as well as iron metabolism markers described previously are assessed though blood sampling. Therefore, the interaction between the blood components mentioned and their interaction with the vascular environment must be acknowledged.

2.4.2) Blood Sampling

Venous blood sampling, typically from the antecubital vein, has been widely used in the collection of blood samples and it is considered the “gold standard” in terms of assessing blood-related parameters. However, it can be an impractical means of sample collection

in resource-poor (MacLennan et al., 2007), field and/or exercise settings (Simmonds, Baskurt, Meiselman, & Marshall-Gradisnik, 2011). Venous blood sampling is not viable in the aforementioned environments since it requires a trained phlebotomist, generates biological waste, can cause discomfort, and may disrupt training (MacLennan et al., 2007; Sitoe et al., 2011).

Micro-sampling, or the use of a decreased volume of capillary blood, has been used as a point-of-care alternative to the collection of venous samples. New mobile analysis equipment has been the focus of a number of studies attempting to demonstrate how results between blood drawn from a finger prick can be interchangeable with results obtained from a venous sample (MacLennan et al., 2007; Sitoe et al., 2011). The former is considered to be minimally invasive, allowing the subject to resume activities with no restrictions. In contrast, a venous blood draw, usually from the antecubital vein, has a no weight bearing criterion in the sampled limb so as to avoid the development of a haematoma and/or soreness.

Studies comparing capillary and venous blood parameters have shown contrasting results. Daae et al. (1988) found that haematocrit, as well as haemoglobin and leucocyte concentrations were significantly higher in capillary samples. In contrast, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were not significantly different between venous and capillary samples. Ponampalam et al. (2012) compared samples in emergency department patients and found statistically significant variations in platelet and haemoglobin concentrations, but acknowledged that such difference had no clinical significance.

The few available studies regarding leucocyte concentration obtained from different sampling sites have provided conflicting results. Ponampalam et al. (2012) found no significant difference in leucocyte concentrations, while Yang et al. (2001) found that total leucocyte concentrations were 9.2% higher in blood samples taken from the fingertip. In particular, concentrations of the large leucocyte populations (mostly granulocytes) showed elevations of 12.6% in capillary samples relative to venous samples. Without specifying populations, Schalk et al. (2007) demonstrated increased leucocyte concentrations in capillary compared to venous samples. When analysing leucocyte populations, Hollis et al. (2012) observed significantly lower lymphocyte concentrations

in capillary samples, while Yang et al. (2001) found no significant difference, further conflicting with an increase of 4.5% for this population found by Daae et al. (1988).

Specific lymphocyte population data are even scarcer. MacLennan et al. (2007) showed that finger-prick blood samples can be used interchangeably with those obtained via antecubital venepuncture for $CD4^+$ lymphocyte counting as a rapid-test alternative for HIV studies. Also in regards to HIV positive patients, Sitoe et al. (2011), using two gold standard flow cytometers, investigated both $CD4^+$ lymphocyte concentration as well as the percentage of total lymphocytes that presented as $CD4^+$ in venous and capillary samples and found close agreement for both (absolute bias=+12.3 cells/mm3, limits of agreement: −259.2 to +283.9, R^2=0.95, p=0.75 and overall bias=+0.6% limits of agreement: −3.1 to +4.3, R^2=0.97, p=0.39, respectively). This demonstrates that the different sample site will not introduce a greater bias than what is already considered acceptable.

Studies comparing leucocyte phagocytic function in different blood vessels are even scarcer. Indeed, the work of Bakhmetyev and Agafonova (2002) is the only study to analyse such variables. When comparing phagocytic activity in arteries, capillaries and veins between healthy men and men with atherosclerosis, they found that neutrophil phagocytic activity in capillary samples was lower compared to arterial and venous blood. They also found that the absolute number of neutrophils in capillary samples was lower than in venous and arterial samples, which contrasts with the findings of Daae et al. (1988) and Yang et al. (2001) as noted above. They also demonstrated elevated monocyte phagocytic activity in capillary samples (Bakhmetyev & Agafonova, 2002).

Despite the diverging results from the few studies available, the variations between capillary and venous blood samples tend to be relatively small and, in most cases, capillary sampling has been suggested to be an acceptable alternative to venous sampling in most cases. However, advances in scientific technology and knowledge of vascular physiology and receptor mediated signalling pathways raise the question if the use of alternate sampling sites is indeed acceptable practice.

2.4.2.1) Vascular Physics and Haemodynamics

The circulatory system connects every system in the body through blood flow. The circulating blood provides every system with the necessary nutrients, O_2 and defence while removing metabolic by-products or waste. Blood is made of plasma and formed elements. Made up of 90% water, plasma carries fundamental solutes such as electrolytes, proteins, nutrients, respiratory gases, and hormones. The formed elements of blood are platelets, erythrocytes, and leucocytes.

The circulatory system is composed of unique blood vessels whose structures are intimately related to their function. Larger vessels such as arteries and veins contain three layers surrounding the vessel lumen: tunica intima, tunica media and tunica externa. Distribution of oxygenated blood through the body is made possible through the arterial system (note that in the pulmonary circulation oxygenation the status of arteries and veins is reversed). Arteries' most distinct layer, the tunica media, is composed of smooth muscle cells and sheets of elastin, allowing control of vascular diameter, and consequently the ability to withstand pressure exerted from the blood leaving the heart. The systemic venular system, responsible for returning deoxygenized blood from the body to the heart, has its most pronounced layer being the tunica externa, mostly composed of longitudinal bundles of collagen fibres and elastic networks. As exchange vessels (O_2, nutrients, immune cells, and waste collection), capillaries are only equipped with the most inner layer, the tunica intima. Even though arteries, veins and capillaries share this common layer composed of endothelium cells (EC), their arrangement and molecular structure vary according to haemodynamic forces suffered according their vascular location.

As a viscous flowing mean, blood has, at any given moment, haemodynamic forces acting on it. These forces are dependent on blood flow (F), pressure (BP), and resistance (R). Blood flow is subject to variation based on vascular mechanical stress caused either by the cyclic pressure of blood hitting vascular walls and/or changes in vessel lumen (vasoconstriction/vasodilation) (Lehoux, Castier, & Tedgui, 2006). This relationship is elucidated by the formula:

$$F = \frac{\Delta BP}{R}$$

where blood flow is directly proportional to differences in blood pressure and inversely proportional to resistance.

Interaction between these three physiological terms generates various types of haemodynamic forces such as hydrostatic pressures, cyclic strains, and wall shear stress (Gimbrone, Topper, Nagel, Anderson, & Garcia-Cardena, 2000). Due to its direct contact with blood, EC alignment, phenotype, gene expression and function are susceptible and adaptive to the shear stress (Aird, 2007; Remuzzi, Dewey, Davies, & Gimbrone, 1984; Resnick & Gimbrone, 1995). This frictional force has shown to, though a series of mechanotransduction-activated pathways (reviewed in Lehoux et al., 2006), increase macromolecular permeability, endothelial cell damage and repair, leucocyte recruitment and expression of adhesion molecules, amongst others (Gimbrone, Nagel, & Topper, 1997; Joris, Zand, & Majno, 1982; Walpola, Gotlieb, Cybulsky, & Langille, 1995).

EC lining the blood vessels were once thought to be static barriers. However, it is now known that though molecular signalling these cells allows passage of not only of water but essential proteins such as albumin. Further, molecular mediated events allow migration of leucocytes through endothelial cells. Inflammatory mediators disrupt the interendothelial junction barrier by binding to their specific receptors leading to a reorganization of the interendothelium thus allowing migration of cells from the blood to the tissue. In true capillaries, this is facilitated due to variations observed in (1) the thickness of the epithelium, being the venular part of the capillary the thinnest section of the circulatory system (0.17μm) as well as in (2) its composition, (e.g. number of vesicles in capillaries (~ $1000/\mu m^3$) is 1.5 and 5.2 times greater than in post capillaries and arterioles, respectively) (Simionescu, 2008). Further, in resting physiological conditions lower shear stress in capillaries and post capillary venules ($\leq$ 0.5 dynes/cm^2) compared to arteries (10-30 dynes/cm^2) (Lipowsky, 1985) allow greater leucocyte-endothelium interaction (Sheikh, Rainger, Gale, Rahman, & Nash, 2003). This was elucidated by the low shear stress activation of CXC chemokines with a glutamate-leucine-arginine (ELR) tripeptide motif (ELR^+CXC chemokine) expression such as IL-8 observed in micro circulation compared to aortic endothelium (Shaik et al., 2009).

Figure 2-7 – Vascular physics

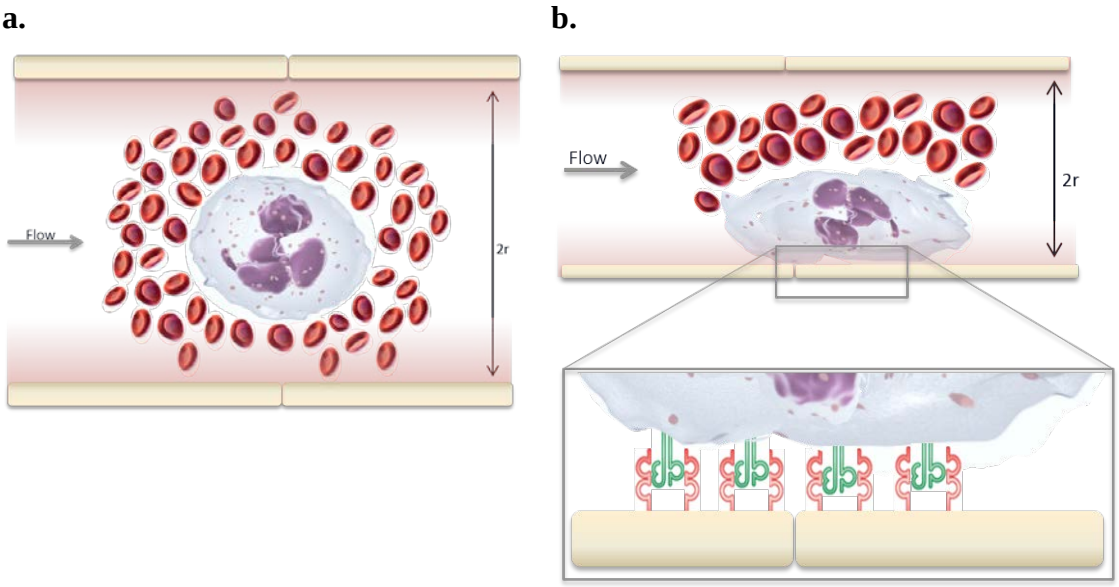


Figure depicts (a) blood flow and interaction of erythrocytes and leucocytes in large diameter vessels e.g. veins and (b) Fahraeus-Lindqvist effect where, in vessels with reduced diameters e.g. capillaries, leucocytes are marginalised towards the vessel endothelium. 'Zoomed-in' section indicates interaction between adhesion molecules on the neutrophilic surface and on the vascular endothelium.

Leucocyte extravasation from blood to tissue is an important process mediating pathogenic clearance. In such, neutrophils' come in contact with the epithelium (rolling) before they eventually adhere and transmigrate. Haemodynamic forces are important determinants of these processes. In decreased diameters, as in capillaries and post capillary venules, preferable sites of neutrophil extravasation, blood flow tends to already facilitate neutrophilic contact with vessel wall as erythrocytes tend to flow towards the centre of the vessel (Fahraeus-Lindqvist phenomenon) (Figure 2-7). Neutrophilic rolling and adhesion has two distinct pathways, a selectin and a CD18-dependent, shown to be dependent on shear rate (Gaboury & Kubes, 1994). L-selectins (expressed by leucocytes) and P-selectins (expressed by platelets and EC) recognize fucosylated carbohydrate ligands expressed on the EC surface. In physiological shear stress, selectins decrease neutrophilic velocity and mediate neutrophilic rolling, as inhibition of such molecules reduced rolling by 90% in vivo (Gaboury & Kubes, 1994). A decrease in neutrophilic velocity in the vessel, exemplified by reductions in blood flow (occlusion) leads to projection on pseudopods – process of cytoplasmic rearrangement – increasing contact with EC prior to migration toward tissue.

If researchers have reached the conclusion that not all endothelium cells are alike and that their molecular heterogeneity is dependent on their location (Aird, 2007), why is it still

assumed that migrating/circulating cells in contact with these different surfaces will behave the same throughout the entirety of the circulatory system?

Micro sampling methods have been introduced in the sporting field as an important tool for evaluating the immediate responses to exercise. To date, these methods have mostly been used for the measurement of blood lactate, glucose, growth hormone and haematocrit (Godfrey, Whyte, McCarthy, Nevill, & Head, 2004). Recently, Simmonds et al. (2011) compared erythrocyte deformability and aggregation in venous and capillary blood using both a finger and earlobe sample, expanding the potential application of this method. During this review of the literature it was not possible to identify a leucocyte micro sampling study including specific lymphocyte population count, presenting values for B cells, T cells and NK cells, or one demonstrating immune function of leucocytes in the sporting field. Therefore, given the conflicting data presented in literature, the need for further validation studies is warranted.

Taken together, the findings highlighted throughout this literature review demonstrate that training evokes short and long-term modulation of both immune response and iron metabolism. Most importantly, the different types of training (i.e. high intensity, hypoxic, high volume) have been shown to determine, in part, the magnitude of the response of immune components, which, in turn, may affect iron status. In almost a cyclic manner, the importance of maintaining adequate iron status is vital for performance and immune competency, particularly in times of high metabolic demands such as increased training intensities or exposure to hypoxia. Immune function and iron status in elite female kayak athletes during different training periods have been largely unexplored. This population presents as a high-risk group for iron deficiency as eumenorrheic females athletes and as participants in mixed energy sport. Compromises in both iron status and immune competency is likely to deter athletes for normal training. Training days lost to illness may, in turn, jeopardise performance. Knowledge of the training-induced modulations of iron status and immune function will provide coaches and sports physiologist with a better understanding of how training regimes affect iron status and immune function and, potentially, performance.

Chapter 3

Methodology

The purpose of this study was to evaluate the modulation of immune and iron status brought about by the adoption of different training periods throughout an Olympic selection year. To overcome issues with current methodologies, a more athlete-friendly method of sample collection was implemented. This method, described and reviewed in section 3.1 below, allowed increased monitoring of the parameters analysed, as it required minimal disruption to training and minimal discomfort to the athletes. A full haematological screening was performed on collected samples using a standard five-part differential haematological Coulter Counter analyser (Beckman Coulter, NSW, Australia). Biochemical analysis was performed using the Cobas INTEGRA® 400 plus multi-analyte analyser (Roche Diagnostics, Switzerland). Enzyme-linked immunosorbent assays (ELISA) were performed on the serum collected to quantify concentration of immune and iron-related variables. Samples were also analysed by flow cytometry to identify alterations in immune populations (phenotyping) and function in accordance with the different training periods under investigation.

3.1) Sample Collection Method

Blood collection was performed using a micro-method. A lancet was used to obtain a micro puncture in the athlete's earlobe. The first drop was wiped away to ensure collection of circulating, not stagnant, blood, free of excess tissue fluids. As a second droplet formed, the tip of the capillary tube touched the droplet allowing blood to flow into the tube through capillary action. Samples dedicated to flow cytometric analysis were collected using lithium heparin as an anti-coagulant in gel separator capillary tubes of 300 µL capacity, while samples used for full blood count were collected in ethylenediaminetetraacetic acid (EDTA)-containing 200 µL capillary tubes. Collection tubes were plastic and, therefore, safe to work with in field situations. Tubes were labelled and kept on ice at 4^{o}C until they were transported to the laboratory at Bond University. Haematological and immunological analysis were performed within two hours of sample collection. Samples for biochemical analysis were centrifuged at 3000 g for 10 minutes, following which plasma was collected and stored at -80^{o}C for subsequent analysis.

A resting venous blood sample was collected prior to and at the end of each training camp utilising a 21-gauge butterfly needle (BD Vacutainer Safety-Lok™, BD Biosciences,

Australia). Samples (4 mL) were collected in EDTA-containing Vacutainer® tubes (BD Biosciences, Australia) for a five-part differential haematological Coulter Counter analyser (Beckman Coulter, NSW, Australia). Samples (5 mL) designated for biochemical analysis (Cobas INTEGRA® 400 plus, Roche Diagnostics, Switzerland) were collected in SST Vacutainer® tube (BD Biosciences, Australia). After being allowed to clot at room temperature for 30 minutes, samples for were centrifuged at 3000 g for 10 minutes, following which 100 µL aliquots of serum were stored at -80^{o}C for subsequent analysis.

3.2) Flow Cytometry

As explicated in the literature review, CDs are commonly used to identify populations with the same phenotypical characteristics as well as determining functional status of a cell. The granulocytic functions explored in the literature review have been associated with expression of receptors on the granulocyte's surface. Once activated, membrane-bound receptors may be up- or down-regulated and as some receptors are located on the surface of granules, the movement of these granules project the receptors to the cell membrane. The receptors may then be quantified with surface staining and flow cytometry.

Flow cytometry is a highly complex technique that allows simultaneous measurement of various physical characteristics of particles (cells) from heterogeneous populations, as they are interrogated by laser beams through a fluid stream. By combining fluidic, optic and electronic systems, flow cytometry provides information on each particle’s size, internal complexity and relative fluorescence intensity.

The fluidics system is responsible for aligning the cells in a single file to be assessed by the optical system. It does so by exploring physical principles of flow systems and coaxial laminar flow dynamic properties as described by Reynolds (1883). Briefly, the sample is injected through a central channel enclosed by an outer sheath containing fluid flowing in higher velocity, creating a drag effect (hydrodynamic focusing) on the central channel. This parabolic profile of flow velocities - created as the central tube is narrowed - positions each individual particle in optimal velocity in the flow cell at the observation region (Picot, Guerin, Le Van Kim, & Boulanger, 2012; Shapiro, 2003).

The optical system of a flow cytometer consists of the illumination (laser), the light collection and detection systems (Ormerod & Imrie, 1990). As the flow of the cell through the pathway of the laser beam interrupts its course, light is deflected, scattered, emitted or absorbed. The size of the cell is determined by the extent of the forward scatter (FSC) while the side scatter (SSC) is proportional to the cell's nuclear structure, cytosolic complexity and granularity. The detection of these properties is independent of fluorescence as the measurement is made mostly from diffracted light detected in the axis of the laser beam (by a photodiode) or from light reflected and refracted at 90° to the laser beam (by photomultiplier tubes – PMT), respectively (Ormerod & Imrie, 1990; Picot et al., 2012; Shapiro, 2003). Combined, the measurements obtained from FSC and SSC provide enough information to differentiate different cell types in a heterogeneous cell population.

Detection of further characteristics in a particle is enabled by coupling of light-excitable molecules (i.e. fluorophores) to a desired specific target, often through receptor-ligand properties. Following the laser interrogation, fluorophores are excited to a higher, yet very unstable, energy state. As the excited molecule cannot withstand such excitation, it loses the recently acquired energy through emission of light at a longer wavelength than the one it was excited by. These wavelengths are detected by fluorescence-exclusive detecting PMTs, which guarantee specificity through filters that allow passage of certain wavelengths while blocking others (Shapiro, 2003). To enhance the possible number of characteristics that may be analysed simultaneously, scientists have developed single and tandem fluorochrome dyes often coupled with antibodies (Recktenwald, 1993). Tandem dye antibodies consist of two conjugated dyes that are covalently linked and whose absorption spectra are in close proximity (30-50 nm). When one of the dyes is excited, its emission is transferred to the nearby dye which then emits its fluorescent signal at a higher wavelength. Multicolour immunofluorescent staining is essential in identifying mixed cell populations or characterizing multiple parameters in single cells by flow cytometry or immunofluorescence microscopy (McCarthy, 2007). Tandem dyes expand the possibilities for fluorescence colour selection of antibodies for use in multicolour flow cytometry. These tandem dyes can provide a much higher wavelength fluorescence emission relative to the excitation wavelength and thus allow for multiple distinct emission ranges from a single laser (Ormerod, 2008).

The collected emissions are then processed by the electronic system by converting the voltage created into digital values. Through a series of voltage amplifiers and analogue to digital converters flow cytometers determine the detector (channel) numbers and plots the values into a linear or logarithmic scale (Picot et al., 2012). Events of interest may be gated allowing further analysis to be made on this specific subpopulation (Rahman, 2006). This enables simultaneous analysis of multiple parameters and subsets contained in a heterogeneous solution (e.g. blood) and helps to better define poorly resolved subpopulations (Recktenwald, 1993).

Based on its ability to analyse rapidly multiple parameters in each individual cell as well as to identify and sort different cell populations, flow cytometry is routinely used in immunological studies. Developed in the late 1960s (Bonner, Hulett, Sweet, & Herzenberg, 1972; Herzenberg et al., 2002; Hulett, Bonner, Barrett, & Herzenberg, 1969), early cytometers measured three parameters: one fluorescent signal and two scatter light signals – FSC and SSC. With technological advances, modern flow cytometers are now able to measure more than 17 fluorescent signals (Perfetto, Chattopadhyay, & Roederer, 2004). Using a FACSVerse™ Flow Cytometer (BD Biosciences, Australia), this study employed a multi-coloured panel to identify the different leucocyte populations, perform a phenotypic and functional analysis of neutrophils, and quantify transferrin receptor expression on leucocytes. The FACSVerse™ Flow Cytometer used contains two spatially separated lasers: (1) a blue Argon-ion laser that emits light at a wavelength of 488nm; and (2) a red diode laser that emits light at a wavelength of 635 nm. The instrument contains four fluorescence channels which are capable of detecting green (FL-1, 515-545 nm), yellow (FL-2, 564-606 nm), orange (FL-3, 653-669 nm) and red (FL-4, > 670 nm) light emissions (BD Biosciences, 2012). Analysis of flow cytometric data files was performed with FACSSuite software (BD Biosciences, Australia). For this study, based on the capabilities of the flow cytometer, the fluorochromes used were as follows:

Table 3-1 – Fluorochromes – maximum excitation and emission wavelengths

Fluorochrome	Excitation Max (nm)	Emission Max (nm)
Fluorescein isothiocyanate (FITC)	494	520
R-phycoerythrin (PE)	496	578
Allophycocyanin (APC)	650	660
*PerCP-Cy™5.5	482	695
*APC-Cy™7	650	785
*APC-H7	650	785
*PE-Cy™7	496	785

* Tandem fluorochromes

3.2.1) Parameters Analysed

3.2.1.1) Immune cell concentration

Immune cell populations were identified using CD antigens with monoclonal antibodies (mAbs) that recognize specific CDs (Kipps, 2010). Each CD was carefully selected to identify a particular cell population (Table 3-2).

Table 3-2 – Leucocyte populations – surface markers

Population	Marker	Distribution
Monocytes	$CD14^+$	Strongly expressed on monocytes
Lymphocytes	$CD3^+CD4^+$	Helper T cell phenotype
	$CD3^+CD8^+$	Cytotoxic T cell phenotype
	$CD3^-CD19^+$	B lymphocyte phenotype
	$CD3^-CD16^+CD56^+$	Natural killer lymphocyte phenotype
Granulocytes	CD11b	Expressed in neutrophils in the metamyelocyte phase (Terstappen, Safford, & Loken, 1990) Complexes with CD18 to form receptor for C3bi, facilitating homotypic or heterotypic adhesion, cell activation, phagocytosis and chemotaxis. Has been used in several studies as marker of granulocyte function (Gray et al., 1993) and has been shown to increase expression up to 75% 12hours after infection (Dosogne et al., 1997).
	CD18	Complexes with several α chains (CD11a-d) and is essential for correct leucocytes adhesion and signalling.
	CD16	FcγRIII found in polymorphonuclear leucocytes; Has been described to be expressed on band (dim) and segmented (bright) neutrophils only (Fujimoto et al., 2000; Terstappen et al., 1990) and described as membrane markers correlating with specific granule and gelatinase (Bainton, 1999).
	CD66b	Expressed exclusively by granulocytes. Glycosylphosphatidylinositol (GPI) isoform that facilitates heterotypic adhesion. Marker of neutrophilic degranulation

Blood was aliquoted (20 µL) into two tubes. CD3 (FITC), CD4 (PE-Cy7), CD8 (PerCP-Cy5.5), CD19 (APC-H7), CD56 (PE) and CD71 (APC) were added to tube one. To the second tube, CD11b (APC-Cy7), CD66b (PerCP-Cy5.5), CD16 (PE), CD18 (FITC) and CD71 (APC) were added. Isotype controls for each antibody were used prior to assay set-up to determine negative and positive populations as per manufacturer's recommendations. All antibodies were purchased from Becton, Dickinson and Company (BD) Biosciences (California, USA). Tubes were incubated for 30 minutes at room temperature in the dark, as fluorochromes are light sensitive. Erythrocytes were lysed with NH_4Cl lysing solution (500 µL). Tubes were incubated for a further 10 minutes at

room temperature in the dark. They were then centrifuged at 300 x g for 5 minutes. Cells were resuspended in phosphate buffered saline (PBS) and fixed with 1% formalin. Samples were analysed through the flow cytometer within two hours of fixation.

3.2.1.2) Immune function

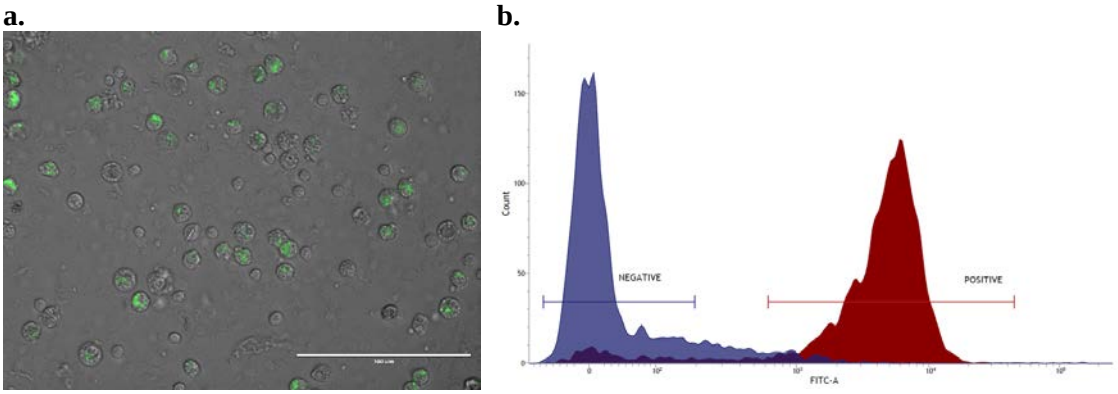
3.2.1.2.1) Phagocytosis

Based on the principle of ingestion of pathogens by phagocytic cells, this assay utilised fluorescently labelled bacteria (FITC labelled *E. coli*) to quantify the percentage of phagocytes that have ingested bacteria and their activity (number of ingested bacteria per cell).

The phagocytic function of neutrophils was analysed in whole blood using a previously described method (Antal et al., 1995; Bohmer, Trinkle, & Staneck, 1992; Hasui, Hirabayashi, & Kobayashi, 1989; Santos, Montes, Gutierrez, & Ruiz, 1995; White-Owen, Alexander, Sramkoski, & Babcock, 1992). Blood was collected in lithium heparin tubes, as anticoagulants such as EDTA and acid citrate dextrose capture Ca^{2+} ions that are essential for phagocytosis (van Eeden, Klut, Walker, & Hogg, 1999). Since the phagocytosis process is greatly dependent on temperature, whole blood (25µL) was aliquoted into two 5 mL tubes labelled 'hot' and 'cold'. Pre-labelled FITC *E. coli* bacteria (5µL) were added to each tube. The 'hot' labelled tube was incubated in a water bath at 37°C for 10 minutes, while the 'cold' labelled tube was placed on ice for 10 minutes. After precisely 10 minutes, the 'hot' labelled tube was placed on ice to stop further phagocytosis. Trypan blue (25 µL) was added to each sample and mixed. This solution quenches the FITC fluorescence of surface bound bacteria, leaving the fluorescence of the ingested bacteria unaltered (Oda et al., 2006). To ensure quenching capacity of trypan blue, visual confirmation of bacterial ingestion by neutrophils was performed in piloted samples of the assay through electronic fluorescence microscopy (EVOS® fl, AMG, Washington, USA) (Figure 3-1a). Tubes were washed with 1mL of PBS and centrifuged for 2 minutes at 1000 x g, after which the supernatant was removed. The washing procedure was subsequently repeated. Following this, erythrocytes were lysed with NH_4Cl lyse solution (500 µL) and incubated for 10 minutes in the dark at room temperature. Samples were centrifuged, washed, and centrifuged again as previously

described. Cells were resuspended in PBS and fixed with 1% formalin. Samples were then analysed at 488 nm on a FACSVerse™ flow cytometer. FSC and SSC determined granulocytic population while histogram distinguished ingestion or not of *E.coli* by granulocytes (Figure 3-1b).

Figure 3-1 – Gating granulocyte phagocytic activity



(a) Electronic microscopy to confirm FITC-labelled *E.coli* ingestion (b) The tube labelled 'cold' was considered negative, while 'hot' tube considered positive. Values for statistical analysis were obtained by the gating the granulocyte population presented on the histogram shown here.

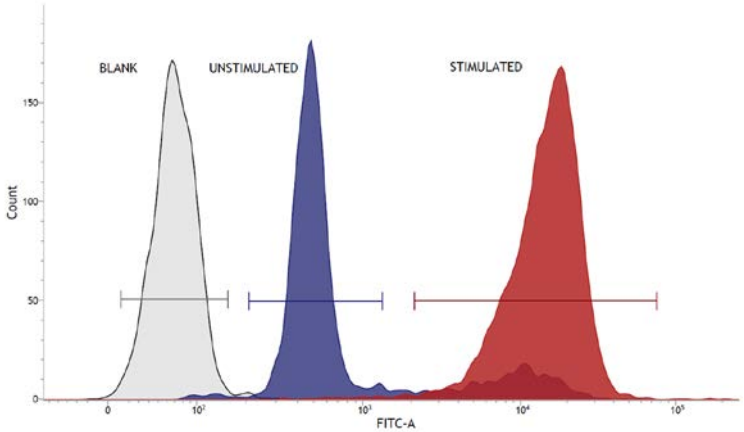
3.2.1.2.2) Oxidative Burst

This test is based on the principle of ROS (particularly H_2O_2) formation of activated granulocytes (i.e. neutrophils) by stimulating the NADPH oxidase system. Physiologically, ROS formation is initiated via phosphorylation NADPH sub-units (Groemping & Rittinger, 2005). IP3-induced Ca^{2+} release into the cytosol of neutrophils will, in combination with diacylglycerol, activate intracellular kinases that activate NADPH oxidase (Bass et al., 1987). Protein kinase C (PKC) has been suggested as the central component of stimulus-response coupling in neutrophils (Bass et al., 1987). Phorbol 12-myristate 13-acetate (PMA) is an analogue of diacylglycerol, binding and activating PKC (Groemping & Rittinger, 2005). PKC then translocates from the cytosol to activate NADPH oxidase and consequently stimulate ROS formation (Bass et al., 1987; Nelson & Cox, 2008). Formation of the reactive oxidants during the oxidative burst can be monitored by the addition and oxidation of dihydrorhodamine 123 (DHR 123) (Rothe, Emmendorffer, Oser, Roesler, & Valet, 1991). Oxidation converts DHR123 to a fluorescent cation, rhodamine 123, which is excited at 511nm and emits fluorescence at

534 nm (van Pelt et al., 1996). The fluorescent signal produced by such oxidation represents mainly H_2O_2 levels (Rothe et al., 1991).

Oxidative burst capacity of granulocytes was analysed in whole blood using a previously described method (Avendano, Sales-Pardo, Marin, Marin, & Petriz, 2008; Richardson, Ayliffe, Helbert, & Davies, 1998; van Pelt et al., 1996; VanderVen, Yates, & Russell, 2009; Vowells, Sekhsaria, Malech, Shalit, & Fleisher, 1995; Walrand et al., 2003; Wan, Myung, & Lau, 1993). Whole blood (25 µL) and PBS (225 µL) were aliquoted into three 5mL tubes labelled 'blank', 'unstimulated' and 'stimulated'. Working solution of DHR123 with a final concentration of 25µg/mL (2.5 µL) was added to the unstimulated and stimulated tubes, mixed and incubated at 37°C for 15 minutes in the dark. Subsequently, a working solution of PMA, with a final concentration of 100ng/mL (2.5µL), was added to the stimulated tube only and mixed. Both DHR123 and PMA were obtained from Sigma-Aldrich (Missouri, USA).All tubes were then incubated once again at 37°C for 15 minutes in the dark, and then centrifuged for two minutes at 1000 x g. The supernatant was then removed. Erythrocytes were lysed with 250 µL of NH_4Cl lyse solution. All tubes were incubated once again at 37°C for five minutes in the dark, before being centrifuged for two minutes at 1000 x g. The supernatant was again discarded. Samples were subsequently washed with 500 µL PBS and again centrifuged for two minutes at 1000 x g following which the supernatant was removed. Finally, samples were resuspended in 125 µL 1% formalin and analysed at 488 nm wavelength on a FACSVerse™ flow cytometer. Figure 3-2 illustrates gating technique utilised using 'blank' tube as control.

Figure 3-2 – Gating granulocyte oxidative burst



Histogram of stimulated tube, with overlayed blank and unstimulated tubes to illustrate gating methodology.

3.2.1.3) Iron studies

3.2.1.3.1) Transferrin Receptor (TfR) CD71

The transferrin receptor (TfR or CD71) is a membrane-bound receptor for the iron-carrying protein, transferrin. CD71 binds serum iron-transport protein transferrin at a neutral pH and iron-free apotransferrin at acidic intracellular pH to facilitate cellular iron uptake (Kipps, 2010). Hence its expression in all active or proliferating cells (Kipps, 2010). Erythrocytes have the greater expression of CD71(Marsee, Pinkus, & Yu, 2010). As this study was based on immunological function, expression of CD71 was measured in the leucocyte populations previously described. This allows for quantification of the requirement for iron by each population, as transferrin receptor expression is known to increase according to intracellular iron requirements.

APC labelled CD71 expression was evaluated through its addition to the immune cell concentration assays. In a multi-coloured panel flow cytometry, the use of the blue and red laser allowed the detection of multiple wavelengths with minimal interference, which was later compensated for. The manufacturer-recommended isotype control (APC IgG_{2a}, κ) was used prior to testing to determine negative and positive populations (Schioppa et al., 2003).

3.3) Enzyme-linked immunosorbent assay (ELISA)

Still exploring the concept of receptor-ligand interactions, this thesis employed the use of enzyme-linked immunosorbent assays (ELISA) to determine concentration of specific proteins and hormones related to immune function and iron metabolism.

ELISA is a commonly used technique originally derived from radioimmune assays published in the 1960s. There are various ELISA techniques described extensively elsewhere (Aydin, 2015; Crowther, 1995). This thesis employed the ‘sandwich assay’(Kato et al., 1977). This technique employs the use of wells pre-coated with primary antibodies to capture the molecule of interest. After incubation, unbound molecules are washed from the sample. Samples are then incubated with a secondary antibody-enzyme complex which binds to the primary antibody-antigen complex. The

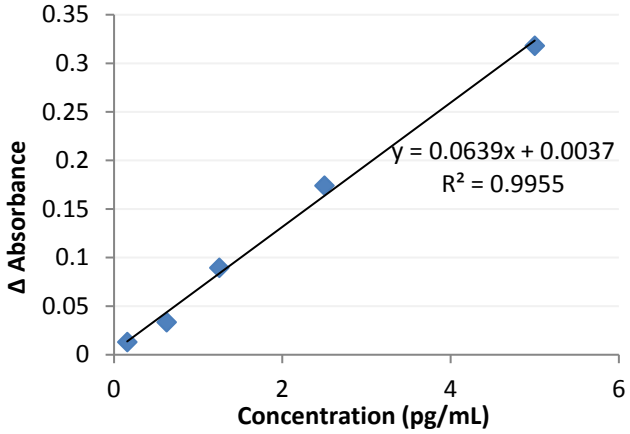
enzyme in the secondary complex the catalyses a colour-producing reaction which can be quantified by measuring absorbance wavelengths (Butler, 1988). The colour intensity is proportional to the concentration of the molecule of interest in the sample.

3.3.1) Parameters analysed

3.3.1.1) Serum myeloperoxidase

The concentration of serum haem-containing enzyme myeloperoxidase, abundant in neutrophils and monocytes, was determined by commercially available enzyme-linked immunoassay (ELISA) (DMYE00B, Quantikine® ELISA – R&D Systems). Briefly, this sandwich enzyme immunoassay contained wells pre-coated with specific MPO monoclonal antibody, where diluted standards or serum samples (1:50) were incubated into for two hours in an orbital microplate shaker set at 500 rpm. After washing procedure, MPO-specific enzyme-linked polyclonal antibody conjugated with horseradish peroxidase (HRP) was added and incubated for two hours as described above. Following washing, wells were incubated with stabilized hydrogen peroxide and chromogen solution for 30 minutes before the addition of a 2N sulfuric acid "stop" solution. The plate was immediately read in microplate reader (iMark, Bio-Rad Laboratories. California, USA) with a 450nm measuring absorbance wavelength with the correction wavelength set at 540 nm. The concentration was determined by plotting absorbance and concentration of known standards in Microsoft Excel and obtaining regression equation (Figure 3-3). The concentration obtained from the standard curve was then corrected appropriately for the dilution factor. Log transformation of data was performed.

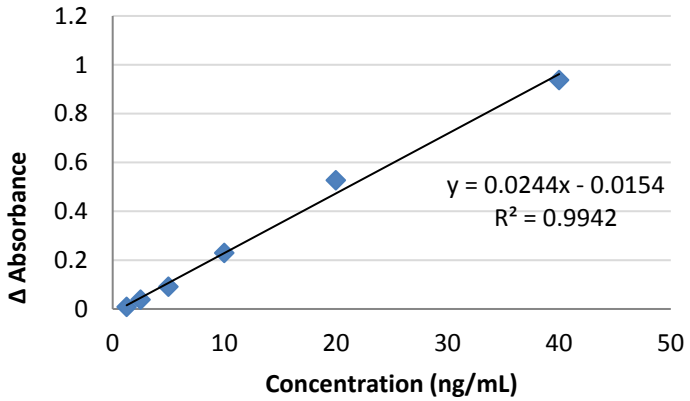
Figure 3-3 – Standards regression for myeloperoxidase ELISA assay



3.3.1.2) Serum lactoferrin

The concentration of LF found in venous-obtained serum was quantified through commercially available ELISA (ab108882- HLF2 Human ELISA Kit – abcam®) with a 0.35 ng/mL sensitivity. Briefly, a 96-well plate containing pre-coated lactoferrin specific monoclonal antibodies was incubated for two hours containing 50 µL of either the standards (prepared as manufacturers' specifications with concentrations ranging from 40 ng/mL to 0.625 ng/mL) or samples (pre-diluted 1:50 with Diluent N provided). A blank well was kept to subtract any noise from the plate. The plate was then washed five times with wash buffer provided before being incubated for one hour with 50 µL of the biotinylated lactoferrin antibody (previously diluted as per instructions provided). Washing was repeated as per instructions, followed by a 30-minute incubation with 50 µL of streptavidin-peroxidase conjugate, after which another wash was performed. Then, 50µL of chromogen substrate were added to each well and incubated for 15 minutes before addition of the "stop" solution. Plate was read immediately in microplate reader (iMark, Bio-Rad Laboratories. California, USA) with a 450 nm measuring absorbance wavelength with the correction wavelength set at 570 nm. The mean values from the duplicate sample wells were calculated based on the standard curve generated through linear regression analysis (Microsoft Excel) with correction to account for the dilution factor (Figure 3-4). Average intra-assay CV was 5.0%.

Figure 3-4 – Standards regression for lactoferrin ELISA assay



3.3.1.3) Serum IL-6

A commercially available sandwich ELISA kit (D6050, Quantikine® ELISA – R&D Systems) was used to quantify the concentration of IL-6 in serum in pre- and post-training samples obtained from capillary and vein of the athletes. Briefly, a 96-well microplate,

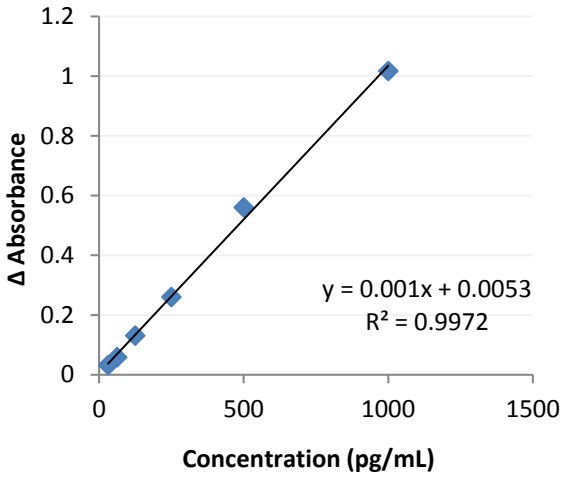
pre-coated with monoclonal antibody specific to human IL-6, was incubated for two hours containing 100 µL of a buffered protein base assay diluent and 100 µL of either standards (serially diluted from 300 pg/mL stock) or samples. Wells were washed four times with wash buffer provided and then incubated for two hours after 200 µL of polyclonal antibody specific for human IL-6 (conjugated with HRP) were added to each well. Washing procedure was repeated and 200 µL of substrate solution (containing stabilized hydrogen peroxide and tetramethylbenzidine (TMB)) was added to each well and allowed to incubate at room temperature, in the dark, for 20 minutes, after which a 2N sulfuric acid 'stop' solution was added to each well. The plate was immediately read in a microplate reader (iMark, Bio-Rad Laboratories. California, USA) with a 450 nm measuring absorbance wavelength with the correction wavelength set at 570 nm. A linear regression standard curve was generated using the mean of the duplicate standards wells (Microsoft Excel). Then, the mean values from the duplicate sample wells were calculated based on the standard curve. Mean intra-assay CV was 1.1%.

3.3.1.4) Serum Hepcidin

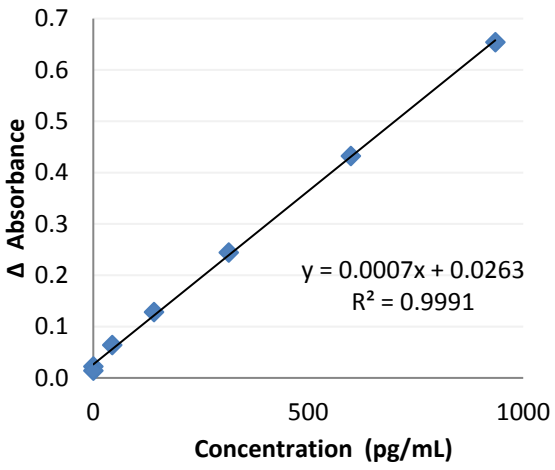
The concentration of venous and capillary-obtained serum hepcidin was quantified separately using sandwich technique commercially available ELISA kit (DHP250 Quantikine® ELISA – R&D Systems) containing 96-well plate pre-coated with a monoclonal antibody specific for human hepcidin. Briefly, 50 µL of assay diluent was added to each well, followed by addition of either standards (7 serial dilutions from 1000pg/mL stock) or diluted samples (1:5). After a two-hour incubation period at room temperature plate was washed (four times) and 200 µL of a monoclonal antibody specific for human hepcidin conjugated to HRP were added to each well. Following another two-hour incubation period, the wash procedure was repeated and 200 µL of a substrate solution containing stabilized hydrogen peroxide and TMB were added to each well and allowed to incubate in the dark for 30 minutes. To interrupt the reaction between TMB and HRP a 2 N sulfuric acid solution was added and the plate was read immediately in a microplate reader set at 450nm with correction set at 540 nm (iMark, Bio-Rad Laboratories. California, USA). Mean intra-assay CV was 2.1% for the capillary samples and 3.0% for the venous samples. The mean values from the duplicate sample wells were calculated based on the standard curve generated through linear regression analysis (Microsoft Excel) with correction to account for the dilution factor (Figure 3-5).

Figure 3-5 – Standards regression for hepcidin ELISA assay

a. Venous serum hepcidin



b. Capillary serum hepcidin



3.4) Biochemical Analysis

The biochemical analysis of iron parameters was conducted through use of a Cobas INTEGRA® 400 plus analyser. Post collection, samples were centrifuged, allowing for the collection of approximately 100 µL of serum. From this minimal volume of sample, all the iron parameters described in section 3.4.1 were measured.

The Cobas INTEGRA® 400 plus biochemical analyser uses light absorbance and microparticle immunoturbidimetric assays to quantify biomarkers in body fluids, such as plasma and serum. Such assays are based on an agglutination reaction induced by antigen-antibody binding. Light is then directed to the sample mixture and the change in absorbance is measured photometrically. The change in absorbance is proportional to the agglutination rate of the microparticle. Biomarkers are measured using competition between the protein contained in the sample and the antigen-coated microparticle for the specific antibody (Koivunen & Krogsrud, 2006).

3.4.1) Parameters Analysed

3.4.1.1) Serum Iron

As previously detailed, iron does not travel freely throughout the body, but rather is bound to transferrin. Serum iron determination is the measurement of the quantity of circulating

iron bound to transferrin. This circulating iron will be carried to the bone marrow to be incorporated in both erythro- and myelopoiesis (Ganz, 2007).

Both physiological and pathological states influence serum iron. Physiologically, serum iron concentration has a diurnal rhythm, which increases and peaks between 7:00-10:00 hours and drops throughout the afternoon reaching its lowest values at approximately 21:00 hours (Beutler, 2010b; Schaap et al., 2013). In females, this parameter fluctuates accordingly to menses (Malczewska et al., 2000). In pathological states such as IDA, acute or chronic inflammation or haemorrhage, serum iron concentrations are decreased (Burtis, Ashwood, & Bruns, 2012). Increased concentrations of serum iron occur in iron-overload disorders such as hemochromatosis, in acute hepatitis, in acute iron poisoning in children, and following oral ingestion of iron medication or parenteral iron administration (Burtis et al., 2012).

The Cobas INTEGRA® 400 plus measured serum iron through the FerroZine method. Fe^{3+} is released from transferrin under acidic conditions (citric acid) and reduced to Fe^{2+} by ascorbate. Bivalent iron ions form a red-coloured chelate complex with FerroZine. To prevent copper interference, cupric ions are bound to thiourea. The colour intensity is directly proportional to the iron concentration in the sample, which is determined by measuring the increase in absorbance at 552 nm (Roche Diagnostics, 2012). This assay has a measurement range of 0.9-179 µmol/L (5-1000 µg/dL).

3.4.1.2) Serum Ferritin

As previously discussed, iron is not found freely in the body as it can be highly toxic. Intra-cellularly, iron is bound to storage proteins such as haemosiderin and ferritin (Deakin, 2000). Ferritin consists of two components, the apoferritin (protein) and the iron core. Iron is deposited within this core as insoluble ferric hydroxide phosphate (Cook, Baynes, & Skikne, 1992). Serum ferritin is the most commonly used single indicator of iron stores (Borch-Iohnsen, 1995). There is a direct correlation between the concentration of ferritin in serum to ferritin stored in the tissues, where 1 µg/L serum ferritin corresponds to 8–10 mg or 120 µg storage iron/kg body weight (Cook, 2005; Finch et al., 1986). However, authors have discussed that as serum ferritin levels increase as part of the acute phase response to inflammation, determining iron status in athletic population

using only ferritin may not accurately portray actual iron storage levels (Nadeem, Shah, Iqbal, Iqbal, & Hanif, 2011; Skikne et al., 2011).

The Cobas INTEGRA® 400 plus measured serum ferritin through a particle enhanced immunoturbidimetric assay, where the precipitate formed from the agglutination of ferritin and latex particles coated with anti-ferritin antibodies is determined turbidimetrically at 552nm (Roche Diagnostics, 2009). The measurement range of the assay was 10-484µg/L (Roche Diagnostics, 2009).

3.4.1.3) Transferrin

Transferrin is a 79 kD glycoprotein containing 679 amino acid residues. It is synthesized mainly by hepatocytes and has a reported half-life *in vivo* of eight days (van Campenhout, van Campenhout, Lagrou, & Manuel-y-Keenoy, 2003). Its role is to transport iron in its redox-inactive form. Iron-loading to transferrin is dependent on pH, temperature, chelator and ionic concentrations (He, Mason, Nguyen, MacGillivray, & Woodworth, 2000). Iron-loaded transferrin delivers iron to active cells through the transferrin-transferrin receptor complex. The complex is internalised and iron is only released once the pH inside the endosome is 5.5, via an ATP-dependent proton pump (Paterson, Armstrong, Iacopetta, McArdle, & Morgan, 1984). The iron-free transferrin molecule is then released back into circulation towards iron-loading sites (i.e. enterocytes, macrophages) where the iron-loading and transport process may by initiated again (Gkouvatsos, Papanikolaou, & Pantopoulos, 2012). Serum concentrations of transferrin range from 25.2-37.8 µmol/L. In a case study, Hayashi et al. (1993) associated the observed stunted growth, anaemia, and increased incidence of infection with transferrin concentrations below 1.26 µmol/L.

Serum concentrations of transferrin were assessed through an automated biochemical analyser (Cobas INTEGRA® 400 plus). Incubated with anti-human transferrin antibodies (rabbit), transferrin formed a precipitate which was determined turbidimetrically at 340 mn. The measuring range of the assay was 1.26-65.5 µmol/L (Roche Diagnostics, 2011b)

3.4.1.4) Soluble Transferrin Receptor

Soluble transferrin receptor (sTfR) is a single polypeptide chain derived from the transferrin receptor. sTfR originates from a proteolytic cleavage between arginine-100 and leucine-101 of the extracellular domain of the transferrin receptor by a matrix metalloproteinase (Speeckaert, Speeckaert, & Delanghe, 2010). Serum sTfR concentration reflects the receptor density on cells and the number of cells expressing receptors; therefore, it is closely related to cellular iron demands and to the erythroid proliferation rate (Infusino, Braga, Dolci, & Panteghini, 2012). In humans, 75% of transferrin receptors are found in erythroid precursors in the bone marrow. When the functional compartment of iron is reduced, the number of transferrin receptors in serum increases, even before the haemoglobin concentration is significantly depressed. Skikne et al., (1990) in a quantitative phlebotomy study aimed at slowly reducing body iron content, demonstrated that serum transferrin receptors remained within the normal range until iron storage has been exhausted. In the same study, the onset of anaemia was observed at soluble transferrin receptor concentrations of 8.8 mg/L (baseline average was 5.3 mg/L).

This study employed the sTfR assay from Cobas INTEGRA® 400 plus which determined sTfR concentrations in serum based on the particle enhanced immunoturbidimetric assay principle. Human soluble transferrin receptor agglutinates with latex particles coated with anti-soluble transferrin receptor antibodies. The precipitate was determined photometrically at 583 nm. The measurement range was 0.5-20.0 mg/L (Roche Diagnostics, 2011a).

3.4.1.5) sTfR/Ferritin Ratio

Serum ferritin levels reflect iron stores while sTfR levels reflect the degree of availability of iron for cells. Calculating the sTfR/log ferritin index from these two measures provides an estimate of body iron over a wide range of normal and depleted iron stores (Beguin, 2003; Skikne et al., 1990). As described by Skikne et al. (2011) the sTfR measurement (expressed in nmol/L or mg/L) is used in conjunction with the ferritin measurement (expressed in ng/mL) to provide a calculated sTfR index using the following equation: $sTfR/\log_{10}$ ferritin. The sTfR/ferritin index thus takes advantage of the reciprocal

relationship between two variables influenced by ID, an increase in sTfR and a decrease in the ferritin concentration. While sTfR and ferritin measurements are useful in the diagnosis of ID and in the differential diagnosis of various types of anaemia, some reports have suggested that the combination of sTfR and ferritin measurements and the calculation of the sTfR/ferritin index improves the ability to accurately classify anaemia, particularly in anaemia accompanying diseases with active inflammation (Punnonen, Irjala, & Rajamaki, 1997).

Studies have shown that ferritin reacts to inflammatory stimuli (Koulaouzidis, Said, Cottier, & Saeed, 2009; Nadeem et al., 2011; Suominen, Punnonen, Rajamäki, & Irjala, 1998). In contrast, sTfR has been shown to be an indicator of ID and is unaffected by concomitant chronic disease and inflammation (Koulaouzidis et al., 2009). The measurement of sTfR/Ferritin index has been described in many studies (Infusino et al., 2012; Malczewska, Szczepanska, Stupnicki, & Sendecki, 2001) as being more accurate and superior to other means of detecting ID. Studies (Skikne et al., 2011) have shown that the combined use of ferritin, sTfR and sTfR/log ferritin as a diagnostic tool more than doubles the detection of IDA, from 41% (ferritin alone) to 92% (three parameters combined) (Table 3-3).

Table 3-3 – Sensitivity of iron markers

	Iron Deficiency Anaemia			**Anaemia of Chronic Disease**		
	Serum Ferritin	sTfR	sTfR/ferritin ratio	Serum Ferritin	sTfR	sTfR/ferritin ratio
Sensitivity	85%	89%	100%	82%	85%	85%
Specificity	64%	81%	81%	78%	100%	97%
Positive Predictive	76%	86%	87%	77%	100%	97%
Negative Predictive	77%	85%	100%	82%	88%	88%
False Positive	36%	19%	19%	22%	0%	3%
False Negative	15%	11%	0%	18%	15%	15%
Accuracy	76%	86%	92%	80%	91%	90%

Adapted from Nadeem et al. (2011)

3.4.1.6) Unsaturated and Total Iron Binding Capacity (UIBC and TIBC)

As previously described, iron is transported through circulation in the ferric form bound to transferrin. Under physiological conditions, only about one third of the iron-binding sites of transferrin are occupied by Fe^{3+}, which represents the average 2.5 mg of iron found in plasma. Measurements of UIBC indicate the additional iron-carrying capacity of transferrin. TIBC, determined by the sum of the serum iron and UIBC, represents the

maximum iron concentration that transferrin can bind (Yamanishi, Iyama, Yamaguchi, Kanakura, & Iwatani, 2003). Serum TIBC varies in disorders of iron metabolism. In IDA, TIBC is elevated and the transferrin saturation is lowered to 15% or less. Low serum iron associated with low TIBC is characteristic of the anaemia of chronic disorders, malignant tumours, and infections (Burtis et al., 2012). In this study, UIBC was determined directly by the FerroZine method (Persijn, van der Slik, & Riethorst, 1971) through a biochemistry analyser (Cobas Integra 400 Plus, Roche Diagnostics). FerroZine binds to unbound excess Fe^{3+} producing a coloured solution. The increase in absorbance measured at 522 nm is directly proportional to the unbound excess iron and indirectly proportional to UIBC (Roche Diagnostics, 2005).

3.4.1.7) Transferrin Saturation

Transferrin saturation (TSAT) is the ratio of serum iron to TIBC. Reductions in iron status cause decreases in transferrin saturation, as circulating iron becomes less available. Under optimum physiological conditions, transferrin is ~30% saturated. A reduction in transferrin saturation below 16% is a reliable index of an undersupply of iron to the developing red cell (Bothwell, Charlton, Cook, & Finch, 1979). However, since iron deficient erythropoiesis occurs not only in ID but also in acute and chronic inflammation or malignant disease, TSAT was used in combination with the above described iron parameters to ensure specificity (Cook et al., 1992). The percentage of transferrin saturation was calculated from the ratio of serum iron to total iron binding capacity multiplied by 100 (%TSAT= [SI/TIBC] x 100)(Fusaro et al., 2005).

3.5) Quantification of Training

Prescription of training intensity is based on five training zones established by the energetic pathway which contributes to ATP throughout the majority of the demanded activity. These zones have been correlated with physiological parameters such as heart rate and $\dot{V}O_{2max}$. The Australian sprint kayak team has an additional three zones which correspond to the demands of the 500m and 200/100m racing, while the last adopted zone is a maximal effort with short duration (5-15 seconds) probably aimed to mimic the power

required to start the race (Table 3-4). The distance accumulated and time spent in each zone determined the intensity of the training camp.

Quantifying training load presents numerous difficulties such as need for expertise, cost and loss of data. Qualitative analysis of training intensity has been proposed and is internationally accepted through a visual scale of rating of perceived exertion (RPE). The commonly adopted accepted Borg Scale translates exercise exertion phrases such as light, hard or very hard to values from 6-20. Such a scale provides data that correlates with measurable physiological parameters such as heart rate ($r>0.80$) and $\dot{V}O_2$, since both increase linearly with work load (Borg, 1970).

Training load encompasses an internal load, where the outcome is measured by the athlete's perception of the load imposed and an external load where the exercise is quantified in either distance covered, velocity or power output. It has been suggested that a combination of the RPE multiplied by the duration of the session yields a reliable, field-based, training load monitoring tool, titled session RPE (Foster et al., 2001; Foster et al., 1995). This method of training load quantification is adopted in a range of both individual (i.e. swimming (Wallace, Slattery, & Coutts, 2009) and team sports (i.e. soccer (Impellizzeri, Rampinini, Coutts, Sassi, & Marcora, 2004)) and has been recently validated in sprint kayak (Borges, Bullock, Duff, & Coutts, 2014). Through the AIS Athlete Management System (AMS) the athletes input training duration, type of training (i.e paddle, running, gym) and RPE for each session. The AMS also provides the athletes with a scale to demonstrate readiness to train at their best (where a comments section allowed any symptoms/illnesses to be reported) and recovery for every session. Data collected from the AMS was used to determine both acute (daily) and cumulative (seven-day rolling average) training load.

Table 3-4 – Sprint kayak training zones

Training Zone	Intensity	Stress Point	RPE	SR · min^{-1}	% HR_{max}	% $\dot{V}O_{2max}$	Lactate (mmol/L)	Blood Lactate Threshold Relationship	Exercise time to exhaustion
T1	Light Aerobic/Recovery	.5	Very light	<60	60 -75	<60	< 2.0	Below LT1	> 3 h
T2	Moderate Aerobic	1.0	Light	56 - 72	75 - 84	60 - 75	1.0 - 3.0	Lower half between LT1 and LT2	1 - 3 h
T3	Heavy Aerobic	2.0	Somewhat hard	70 - 82	82 - 89	75 - 85	2.0 - 4.0	Upper half between LT1 and LT2	20 min to 1 h
T4	Anaerobic Threshold	3.0	Hard	78 - 92	88 - 93	85 - 90	3.0 - 6.0	LT2	12 - 30 min
T5	Maximal Aerobic (1000m race pace)	5.0	Very Hard	90 - 100	92 - 100	90 -100	> 5.0	Above LT2	5 - 8 min
T6	500m race pace	8.0	Very, very hard	106 - 120	100	n/a	> 8.0	Above LT2	1.5 - 2 min
T7	200m race pace	11.0	Almost maximal	115 - 140	-	n/a	> 6.0	Above LT2	30 - 50 sec
T8	Sprints	15.0	Maximal	> 130	-	n/a	-	-	10 - 15 sec

RPE- Rate of perceived exertion SR-Stroke Rate HR – Heart Rate LT- Lactate Threshold. Source: Bullock, Woolford, Peeling, & Bonetti (2013)

Study I

Chapter 4

Comparison of the use of capillary and venous blood in the analysis of concentration and function of leucocyte sub-populations

Canetti, E.F.D; Keane, J.; Gray, B.

Introduction

Physiological systems and biochemical processes are assessed routinely through a range of techniques that utilise blood samples. Venous blood sampling, typically from the antecubital vein has been widely used and is considered the "gold standard" in the collection of blood samples. However, it can be an impractical mean of blood sampling in resource-poor (MacLennan et al., 2007), field and/or exercise settings (Simmonds et al., 2011) since it is relatively invasive, requires a trained phlebotomist, generates biological waste, can cause discomfort and may disrupt training (MacLennan et al., 2007; Sitoe et al., 2011). Alternatively, micro-sampling, or the use of a decreased volume of capillary blood, has been used as a "point-of-care" means to obtain a blood sample. Micro-sampling is considered to be minimally invasive and allows the subject to resume activities with minimal restrictions, and thus is ideal in the aforementioned environments.

Mounting evidence demonstrates a transient but significant immune suppression following exercise (Nieman, 1997a). As most of the immune components are circulating through blood, it has come to the attention that new methods of blood collection may be warranted to allow increased sampling time points and consequently a more detailed analysis of the immune response to exercise. Even though leucocyte concentration is routinely tested, temporary functional alterations, particularly of neutrophils, are more frequent and are largely neglected in the clinical practice (Hoang et al., 2013). Micro-sampling methods have been introduced in the sporting field as an important tool for monitoring immediate responses to exercise. However, to date, the use of these methods has been limited to blood lactate, glucose, growth hormone and haematocrit profiling (Godfrey et al., 2004).

Whilst it might be assumed that venous and capillary samples would yield identical results, emerging evidence suggests otherwise. Although erythrocyte parameters such as haematocrit and haemoglobin concentration, have been found to be significantly higher in capillary samples (Daae et al., 1988), leucocyte concentrations have provided conflicting results. Capillary samples have yielded increased (Schalk et al., 2007; Yang et al., 2001), decreased (Bakhmetyev & Agafonova, 2002) or not significantly different (Ponampalam et al., 2012) results for the concentration of leucocytes when compared to venous samples. Furthermore, analysis of leucocyte subpopulations in capillary samples has also produced diverging results, where lymphocyte concentration has been found to

be increased (Daae et al., 1988), decreased (Hollis et al., 2012) or unaltered (Yang et al., 2001) in comparison to results obtained from venous samples. Yang et al. (2001) demonstrated a 12.6% increase in the concentration of granulocytes from capillary samples, while Hollis et al. (2012) and Rao, Moiles and Snyder (2011) suggested that capillary and venous samples could be used interchangeably for this cell population.

In addition to the concentrations of specific leucocyte subpopulations, an individual's immunological status relies on proper functioning of these sub-populations. Neutrophils, the most abundant leucocyte in the peripheral circulation, provide defence from pathogens through a series of microbicidal activities. Molecular interactions between neutrophilic surface receptors and inflammatory signals, such as chemokines and cytokines, trigger a chain of events including rolling, adhesion, chemotaxis, phagocytosis and degranulation, which provides effective pathogenic clearance (Futosi, Fodor, & Mocsai, 2013; Kobayashi & DeLeo, 2009). Studies comparing leucocyte function in different blood vessels are rare. Bakhmetyev and Agafonova (2002) was the only study found to date to analyse such variables. When comparing phagocytic activity in arteries, capillaries and veins between healthy men and men with atherosclerosis, these researchers found that neutrophil phagocytic activity in capillary samples was significantly lower compared to cells obtained from arterial and venous blood.

In light of such inconsistent results in the literature, it is reasonable to assume that different vascular regions may impart different biochemical and biophysical forces on the cell. The most notorious difference in vascular anatomy is the calibre of the different vessels. Forces such as flow and shear stress are directly influenced by vascular diameter (Pyke & Tschakovsky, 2005). As the internal diameter of blood vessels varies from 3.0cm in abdominal aorta (Erbel & Eggebrecht, 2006) to 4μm in arterial capillaries (Braverman, 2000), it would be imprudent to neglect the biofluid mechanics of the forces acting on blood cells as they pass through ever-changing vessel diameters. Previous diverging results in leucocyte concentration and function may not have taken into account that the difference in diameter of the vessels may exert different forces onto the flowing cells. Such changes in vascular diameter impact the flow of cells and consequently the interaction between the blood components. Erythrocytes flow through the centre of the vessel marginating leucocytes and platelets towards the vascular epithelial (Sundd, Pospieszalska, & Ley, 2013). This event, termed the "Fahraeus-Lindquits phenomenon", is known to produce a more pronounced haematocrit in samples obtained from the

microcirculation compared to larger vessels (Papaioannou & Stefanadis, 2005). As the majority of neutrophil functions are receptor-mediated events, it is then reasonable to cogitate that any alteration in the interaction of blood components with the vascular endothelial may impact on leucocyte function.

Polychromatic flow cytometry analysis has allowed recognition of unique antigens expressed on the surface of each particular cell population. Some of these antigenic structures are also markers of specific cell functions and may be up-regulated or down-regulated as necessary. This study employed common cluster of differentiation (CD) antigens for identification of monocytes, granulocytes, lymphocytes and their subsets. Cells were identified with anti-human monoclonal antibodies as helper T lymphocytes (expressing $CD3^+$ $CD4^+$), cytotoxic T lymphocytes ($CD3^+$ $CD8^+$), B lymphocytes ($CD3^-$ $CD19^+$), natural killer (NK) cells ($CD3^-CD56^+$) and monocytes ($CD14^+$). Granulocytes were labelled with antibodies against specific antigenic structures that relate to neutrophil developmental stage and distinct neutrophilic functions (CD66b, CD16b, CD11b and CD18). Owing to the functional relevance of these granulocyte surface antigens (Elghetany, 2002; Kuijpers et al., 1991; Lund-Johansen & Terstappen, 1993; Schmidt et al., 2012; Wang et al., 2013) the level of expression of these markers was also assessed. Thus, profiling such surface markers may elucidate developmental distribution of neutrophils as well as possible alterations in functional capabilities according to vascular location.

Given the conflicting data presented in literature, the need for further validation studies is warranted. During the review of literature associated with this study it was not possible to identify previous studies in which a micro sampling technique was applied to the investigation of specific lymphocyte population concentration, presenting values for B cells, T cells and NK cells, and granulocyte phenotypes, as well as functionality of neutrophils. Therefore, using a combination of light scatter properties and specific antibody labelling, this study aimed to produce a comprehensive phenotypic profile of clinically important leucocyte subsets and assess neutrophilic function in venous and capillary sites. It was hypothesised that blood sampling site would yield statistically different results for leucocyte concentration. Further, surface antigen expression and neutrophilic functions of phagocytosis and oxidative burst were hypothesised to produce statistically different results between blood sampling sites. Validation of the technique of capillary sampling opens up the field of exercise immunology to previously inaccessible

field-based research. Furthermore, a greater number of sampling time points will allow for increased immune function monitoring, which may, in the future, be used as a tool to prescribe individually tailored training loads aiming to attenuate or even avoid immune suppression post exercise.

Methodology

Participants

A total of ten healthy volunteers (age: 25.1±3.1 years; height: 174.8±7.3 cm; mass: 73.3±8.9 kg), with males and females in equal number, were recruited via electronic correspondence from Bond University, Gold Coast, Australia. Subjects were instructed to attend a single appointment at Bond University, between 9-11am. Informed consent was obtained as per Bond University Ethics committee requirements.

Blood Collection

Upon arrival, subjects were requested to rest in a seated position for approximately 20 minutes before blood collection. Topical cream containing nonivamide (1.7 mg) and butoxyethyl nicotinate (10.8mg) (Finalgon® Boehringer-Ingelheim, Reims, France) was applied to one earlobe and the hand (opposite to venous blood collection) was placed into warm water (37-38^{o}C) for five minutes to encourage blood flow to the capillary sites. The venous blood sample was collected first to allow for both cream and warm water to increase blood flow in the earlobe and fingertip, respectively. A venous blood sample was drawn from the antecubital vein using a 21 gauge butterfly needle (BD Vacutainer Safety-Lok™, BD Biosciences, Australia) into a 4mL heparin Vacutainer® tube (BD Biosciences, Australia), followed by immediate collection into a 4mL EDTA Vacutainer® tube (BD Biosciences, Australia). Following venous blood sampling, the hand was dried and capillary blood from fingertip was collected using a 2 mm contact activated lancet (BD Biosciences, Australia) to the lateral portion of distal phalanx of third or fourth metacarpal into a 300 µL heparinised capillary tube (Kabe Labortechnik, Germany) immediately, followed by collection 300 µL EDTA capillary tube (Kabe Labortechnik, Germany). Earlobe blood collection followed the same procedures. An alcohol swab was used to remove any cream excess. The inferior border of earlobe was punctured using a 2 mm contact activated lancet (BD Microtainer® Contact-Activated

Lancet BD Biosciences, Australia) and blood was collected into a 300 μL heparinised capillary tube immediately, followed by collection into a 300 μL EDTA capillary tube (Kabe Labortechnik, Germany). The first blood drop for both fingertip and earlobe was discarded to minimize excess tissue fluid. All tubes were placed onto a roller (Roller Mixer SRT6, Stuart) and analysed within two hours of collection. All blood measurements and analyses were performed by a single investigator.

Full Blood Concentration

Samples collected using EDTA as an anti-coagulant were analysed in a five-part differential haematological analyser (Beckman Coulter, NSW, Australia) immediately after collection, for the determination of total leucocyte concentration, specific differential leucocyte concentration (neutrophils, eosinophils, basophils, lymphocytes and monocytes), erythrocyte concentration, haemoglobin, haematocrit, mean corpuscular volume (MCV) mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and red cell distribution width (RDW).

Leucocyte Phenotyping

Whole blood (capillary site 25 μL; venous site 100 μL) was aliquoted into two 5 mL tubes (BD Falcon™ Tubes) containing previously aliquoted monoclonal antibodies with concentrations as per manufacturer's instructions for venous tube and adjusted proportionally for capillary tubes (Table 4-1). Tubes were incubated for 15 minutes at room temperature in the dark. Erythrocytes were lysed with ammonium chloride (NH_4Cl) solution (933-001-1, Kinetik, Australia) for further 10 minutes under the same conditions. Samples were then centrifuged at 300 x g for 5 minutes, washed and centrifuged at 300 x g for 5 minutes once more. Cells were resuspended in PBS (300μL) and analysed immediately.

Phenotypical Analysis

A FACSVerse™ flow cytometer equipped with a two argon-ion lasers, 488nm blue laser and a 640nm red laser was used to excite fluorochromes presented and data were collected into FACSuite 5.0 software (BD Biosciences, Australia). Acquisition was set to 10,000 total events and manual gates were set on the three major populations (lymphocytes, monocytes and granulocytes) using forward and side-scatter properties. Median

fluorescence intensity was taken as an index of antigen expression and was recorded. All measurements were performed after the instrument passed quality control using CS&T Beads (BD Biosciences, Australia) and analysed under fixed instrument settings (photomultiplier tube voltages and compensations).

Table 4-1 – Monoclonal anti-human antibodies (mAb)

Tube	mAb	Clone	Fluorochrome	Concentration	Catalogue	Lot
1	CD3	HIT3a	FITC	1 µg/20 µL	555339	3035946
	CD4	SK3	PE-Cy™7	0.015 µg/5 µL	557852	3060697
	CD8	RPA-T8	PerCP-	0.125 µg/5 µL	560662	3179537
	CD19	SJ25C1	APC-H7	0.5 µg/5 µL	560177	3193985
	CD56	B159	PE	0.125 µg/20	555516	2317618
	CD71	M-A712	APC	0.03 µg/20 µL	551374	3060509
2	CD11b	ICRF44	APC-Cy™7	1 µg/5 µL	557754	3256562
	CD18	L130	FITC	6.25 µg/mL	347953	3199505
	CD16b	CLB-	PE	0.25 µg/20 µL	550868	3329800
	CD66b	G10F5	PerCP-	0.125 µg/5 µL	562254	3193504
	CD14	M5E2	PE-CY™7	0.25 µg/5 µL	557742	3011778
	CD71	M-A712	APC	0.03 µg/20 µL	551374	3060509

All fluorochromes were purchased from Becton Dickinson (BD) Bioscience. FITC (fluorescein isothiocyanate); PE (R-phycoerythrin); APC (allophycocyanin); APC-Cy™7 (APC-cyanine tandem fluorochrome), PerCP-Cy™5.5 (PerCP-cyanine tandem fluorochrome); APC-H7 (APC-cyanine tandem fluorochrome); PE-Cy7 (PE-cyanine tandem fluorochrome)

Granulocyte Function

Phagocytosis

Whole blood (capillary site 25 µL; venous site 100 µL) was aliquoted into two 5mL tubes (BD Falcon™ Tubes) labelled ‘hot’ and ‘cold’. Pre-labelled FITC *E. coli* bacteria (concentration of 2 x 10^7 per 100µL of whole blood) was added to each tube. The ‘hot’ labelled tube was incubated in a water bath at 37°C for 10min, while the ‘cold’ labelled tube was placed on ice for 10 minutes. After precisely 10 minutes, the ‘hot’ labelled tube was placed on ice to stop further phagocytosis. Trypan blue (12.5µL and 50µL, respectively) was added to each sample and mixed to quench any bacteria attached to cell

surface and consequently ensure that the fluorescence observed was from internalized (phagocytosed) *E.coli* only. Tubes were washed with PBS and centrifuged for 2 minutes at 1000 x g, after which the supernatant was removed. The washing procedure was repeated. Erythrocytes were lysed with NH_4Cl solution (933-001-1, Kinetik, Australia) and incubated for 10 minutes in the dark at room temperature. Samples were centrifuged, washed and centrifuged again as described above. Cells were resuspended in PBS and analysed using an excitation wavelength of 488nm on a FACSVerse™ flow cytometer (BD Biosciences, Australia).

Oxidative Burst

Oxidative burst function of neutrophils was analysed in whole blood using a previously described method (Richardson et al., 1998). Briefly, this method quantified the production of ROS, principally H_2O_2, in response to activation of the NADPH oxidase system, stimulated by a protein kinase C activator and an analogue of diacyglycerol, phorbol 12-myristate 13-acetate (PMA) (Nauseef, 2014; Groemping and Rittinger, 2005). ROS production was quantified by the oxidation of the dye dihydrorhodamine (DHR), by hydrogen peroxide, which results in a fluorescence emitting compound (530-535nm), rhodamine 123 (Richardson et al., 1998). Whole blood (capillary site 25µL; venous site 100µL) and PBS (225µL and 900µL respectively) was aliquoted into three 5mL tubes (BD Falcon™ Tubes) labelled 'blank', 'unstimulated' and 'stimulated'. DHR (final concentration 25ug/mL) was added to the unstimulated and stimulated tubes, mixed and incubated at 37°C for 15min in the dark. PMA (final concentration 100ng/mL) was added to the 'stimulated' tube only and mixed. All tubes were then incubated once again at 37°C for 15min in the dark, and then centrifuged for 2 minutes at 1000 x g. Erythrocytes were lysed using a NH_4Cl solution (933-001-1, Kinetik, Australia) for 10 minutes at room temperature in the dark, before being centrifuged for 2 minutes at 1000 x g. Samples were washed with 500µL PBS and again centrifuged for 2 minutes at 1000 x g. Finally, samples were resuspended in 300µL 1% formalin and analysed using an excitation wavelength of 488nm wavelength on a FACSVerse™ flow cytometer (BD Biosciences).

Statistical Analysis

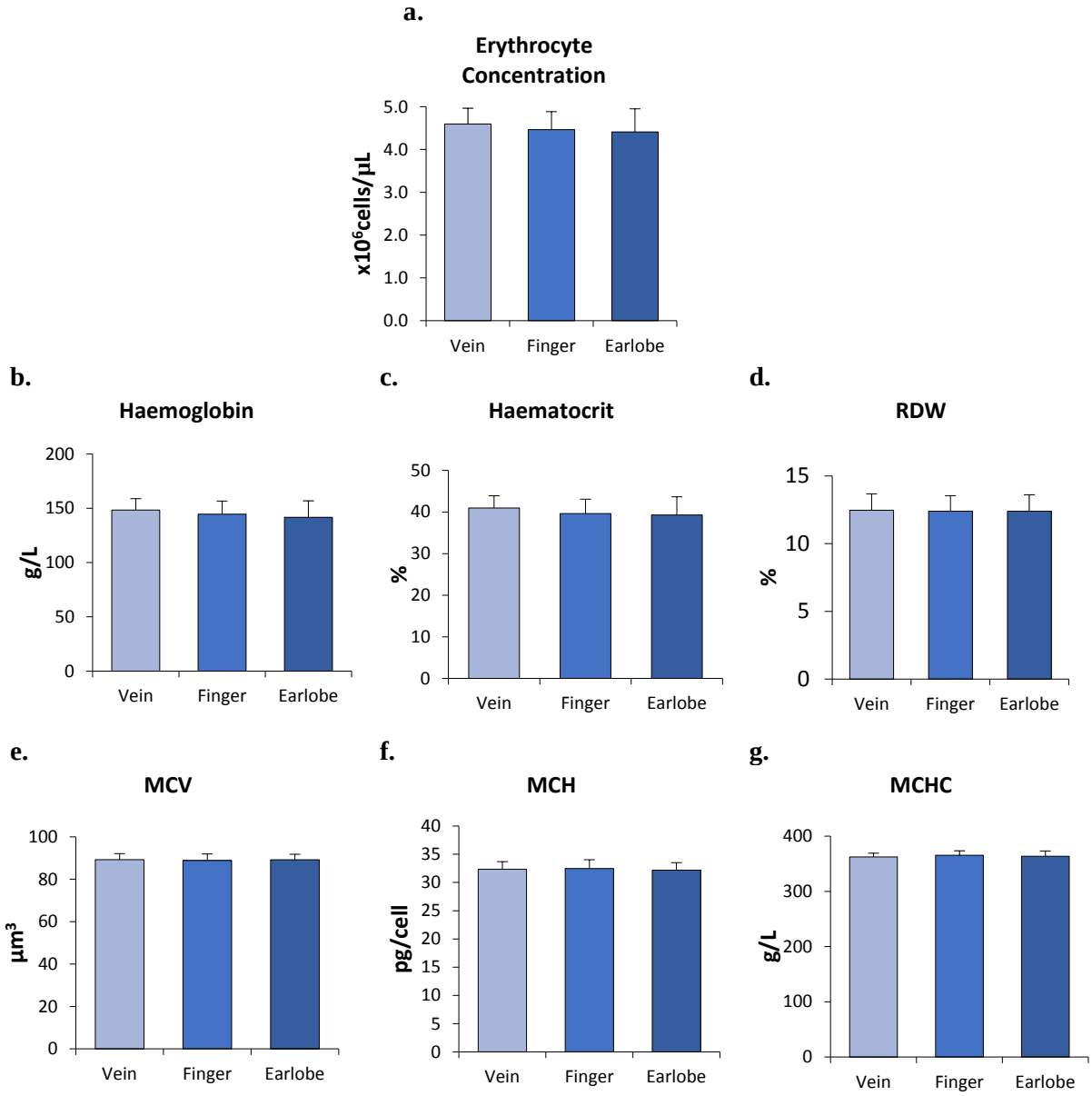
A multivariate analysis of variance (MANOVA), utilizing Pillai's Trace with $\alpha \leq 0.05$ significance level, analysed the effects of the different sampling sites in the selected erythrocyte parameters, the main leucocyte populations, and the CD expression in each leucocyte population. MANOVA was also used in all granulocyte functional analysis. Prior to the MANOVA data was examined using SPSS Statistics to ensure all underlying assumptions were met. MANOVA was chosen since it has been described to have greater power than ANOVA to detect effects because it takes into account the correlations between dependent variables (Field, 2009). MANOVA was reported as $V = , F(,) = , p < .05.$, where V is the result of the Pillai's Trace, F is the ratio of the model to its error, and, in between brackets, are the degrees of freedom, hypothesis and error, respectively (Field, 2009). In the event of a significant difference being identified, the post-hoc Bonferroni test was used as well as a discriminant function analysis to verify how the dependent variables discriminate the groups. All statistical analyses were performed using SPSS (IBM SPSS Statistics for Windows, version 20; IBM Corp., Armonk, N.Y., USA).

Results

Erythrocyte Parameters

Erythrocyte parameters analysed showed no significant differences between vein, finger, and earlobe sampling sites (p=0.526) (Figure 4-1 a-g).

Figure 4-1 – Erythrocyte parameters analysed

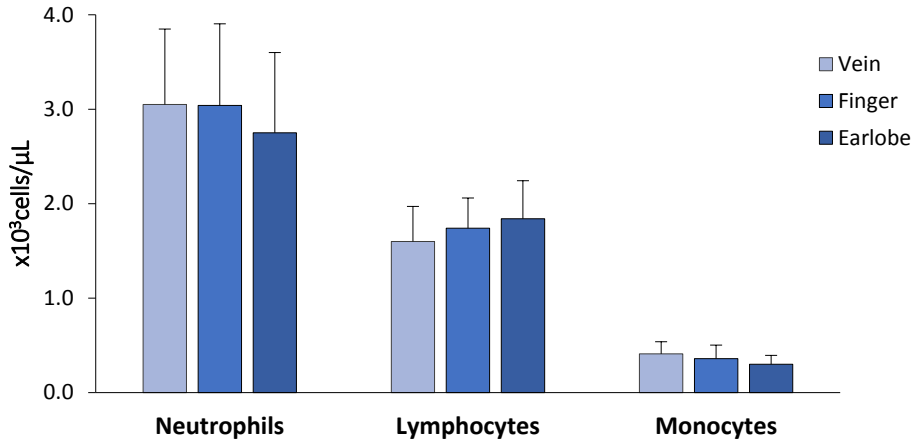


Erythrocyte parameters analysed: (a) erythrocyte concentration (b) haemoglobin (c) haematocrit (d) red cell distribution width (RDW) (e) mean corpuscular volume (MCV) (f) mean corpuscular haemoglobin (MCH) (g) mean corpuscular haemoglobin concentration (MCHC). Solid bars represent mean values and error bars represent SD. V=0.460 F(14,44)=0.940 p>0.05.

Leucocyte Concentrations

Concentration of total leucocytes and of its specific populations (neutrophils, eosinophils, basophils, lymphocytes, and monocytes), showed no significant difference between the three sampling sites (p=0.447), as shown in figure 4-2.

Figure 4-2 – Leucocyte concentration

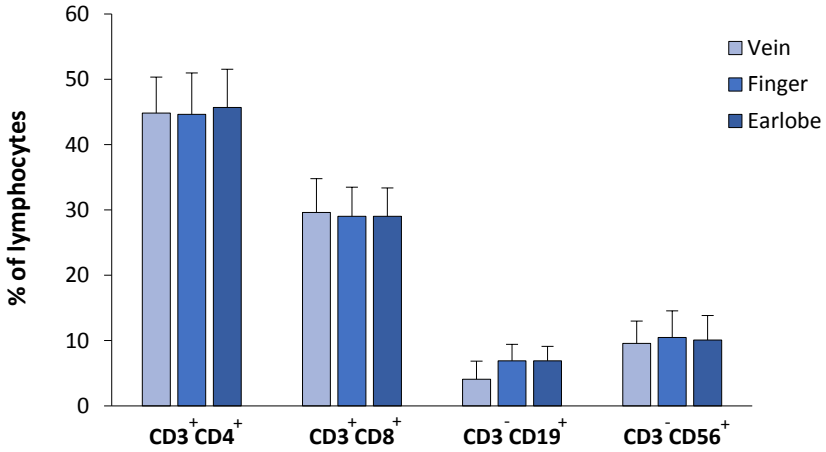


Leucocyte concentration. Solid bars represent mean values and error bars represent SD. Eosinophil and Basophil populations were not depicted in the figure as numbers are low in healthy volunteers. Mean ± SD for eosinophil population in vein, finger and earlobe are 0.11 ± 0.09, 0.12 ± 0.09 and 0.08 ± 0.07, respectively. Mean ± SD basophil population in vein, finger and earlobe are 0.0 ± 0.00, 0.0 ± 0.00, and 0.0 ± 0.00, respectively.

Lymphocyte Subpopulations

Lymphocyte populations showed no significant difference in phenotypical distribution amongst the three sampled sites (p=0.241) (Figure 4-3).

Figure 4-3 – Phenotypical distribution of lymphocyte subpopulations

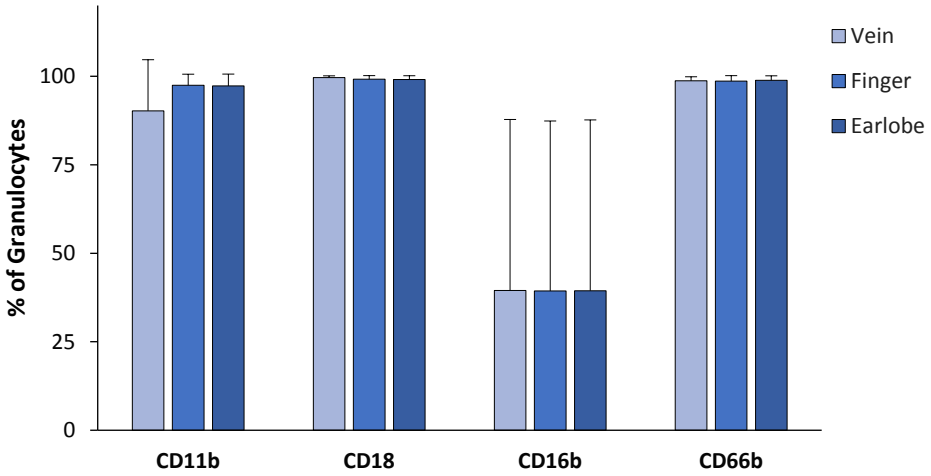


Phenotypical distribution of lymphocyte subpopulations based on known surface antigen expression. Solid bars represent mean values and error bars represent SD. V=0.368 F (8, 48) = 1.355 p > 0.05.

Granulocyte Subpopulations

Percentage of cells positive for granulocytic markers showed no significant difference between the sampling sites (*p=0.237*) (Figure 4-4).

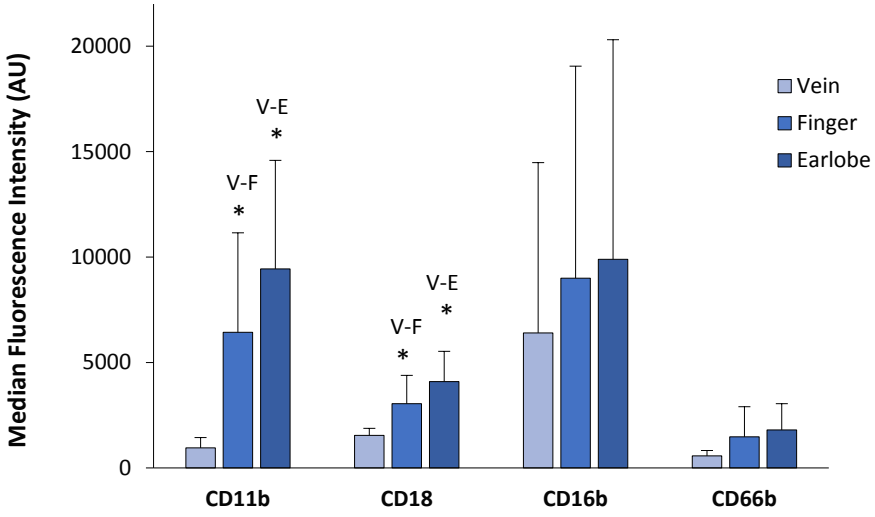
Figure 4-4 – Phenotypical distribution of granulocytes



Phenotypical distribution of granulocytes. Solid bars represent mean values and error bars represent SD. *V*=0.358 F (8, 50) = 1.361 *p*>0.05. Subjects not expressing CD16b (Clone CLBgran11.5) are considered zero.

However, surface receptor expression between granulocyte subpopulations utilizing Pillai's trace showed significant difference between sites (*p=0.008*), where a Bonferroni post-hoc test demonstrated significant differences between venous and capillary samples for both CD11b (Vein-Finger *p=0.023* / Vein-Earlobe *p=0.000*) and CD18 (Vein-Finger *p=0.030* / Vein-Earlobe *p=0.000*) markers (Figure 4-5).

Figure 4-5 – Phenotypical expression of granulocyte phenotypes

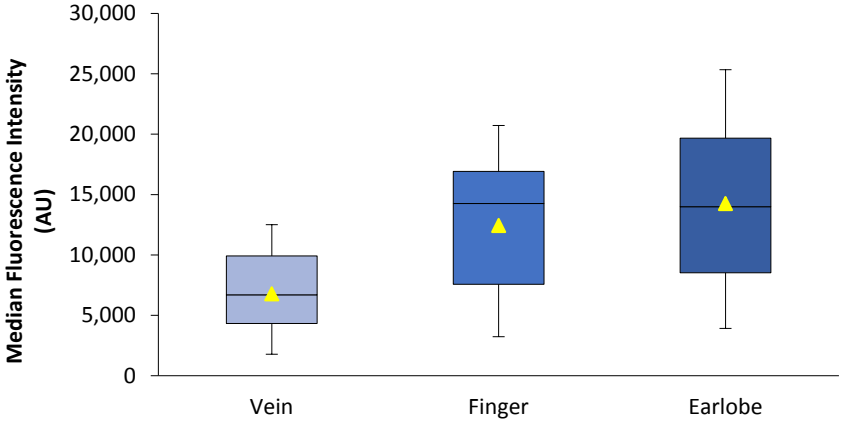


Phenotypical expression of granulocyte phenotypes measured through median fluorescence intensity. Solid bars represent mean values and error bars represent SD. V=0.646 F (8, 50)=2.98 *p*<0.05. * Significant difference between vein and capillary samples.

Granulocyte Function

Multivariate analysis assessing difference between sampling sites in regards to percentage of total granulocytes, percentage of ROS producing granulocytes and ROS production (measured as median fluorescence intensity – Figure 4-6) showed no significant difference between sampling sites (*p=0.198*)

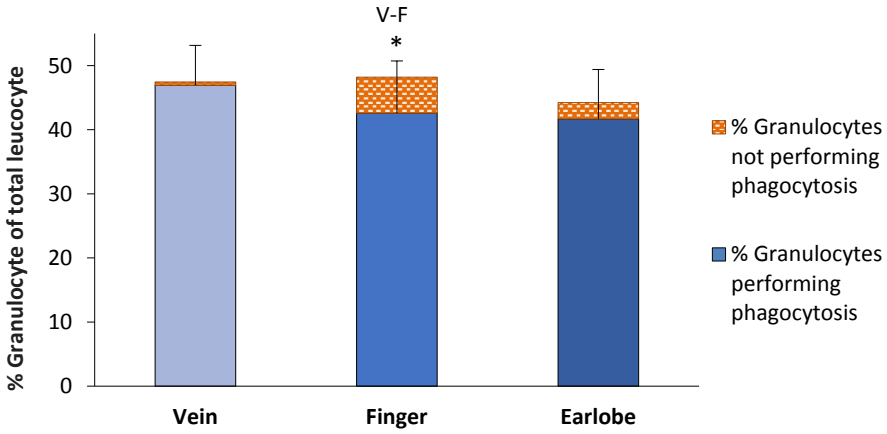
Figure 4-6 – Stimulated ROS production by granulocytes



Granulocytes producing ROS. Solid bars represent mean values and error bars represent SD. *V*= 0.305 F (6,50)= 1.498 *p*>0.05.

Multivariate analysis of the percentage of granulocytes in each sample, the percentage of granulocytes performing phagocytosis and amount of *E.coli* ingested (measured by median fluorescence intensity) demonstrated a significant difference between the three sampling sites (*p=0.011*). Bonferroni post-hoc analysis indicated a difference in percentage of granulocytes carrying out phagocytosis between vein and finger sample (*p*=0.025), but not between vein and earlobe (*p*=0.638) or finger and earlobe (*p*=0.389).

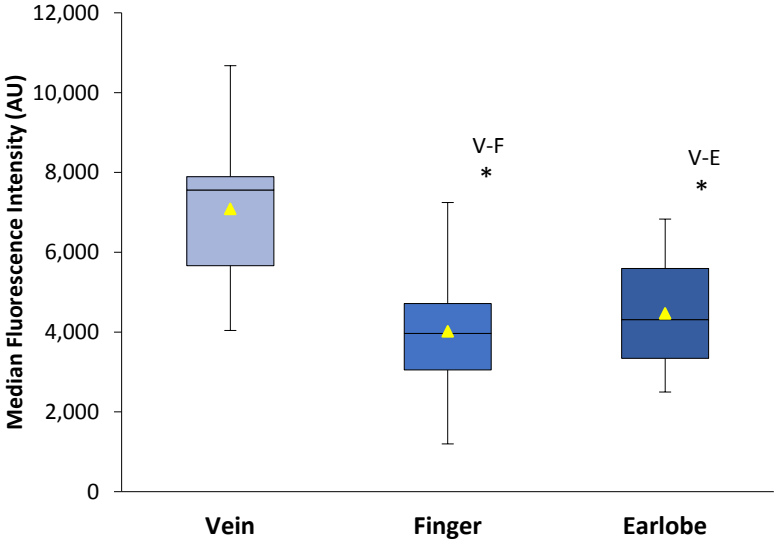
Figure 4-7 – Granulocytes phagocytosing *E.coli*



Granulocytes phagocytosing *E.coli*. *V*=0.527 F (6,52) = 3.099 *p*<0.05. Solid bars represent mean values. The error bars represent SD of the percentage of granulocytes performing phagocytosis. (*) Significant difference in the percentage of cells performing phagocytosis between assigned sites.

Furthermore, the amount of *E.coli* ingested was significantly different between vein and finger (p=0.001), between vein and earlobe ($p=0.006$) but not between finger and earlobe (p=1.000) (Figure 4-8).

Figure 4-8 – *E.coli* ingested by granulocytes according to sampling site



E.coli ingested by granulocytes, measured by MFI. V=0.527 F (6,52)=3.099 $p<0.05$. Solid bars represent mean values and error bars represent SD. (*)Significant difference in the number of *E.coli* ingested between the sites.

Discussion

This study was conducted to compare leucocyte distribution, phenotypical expression of specific antigens in particular leucocyte subpopulations, as well as granulocytic (i.e. neutrophils) phagocytic and ROS producing functions between three blood collection sites (vein, finger and earlobe). It was hypothesised that erythrocyte parameters (Figure 4-1a-g) and leucocyte distribution (Figure 4-2) would be significantly different between the three different sampling sites. Statistical analysis did not confirm this hypothesis. Distribution of lymphocyte subpopulations (Figure 4-3) and of granulocyte subpopulations (Figure 4-4) showed no significant difference between the sampling sites. However, further analysis of surface receptor expression in granulocytes, particularly CD11b and CD18 (Figure 4-5) revealed significant differences between the venous and capillary sampling sites. While granulocytic, PMA stimulated, ROS production showed no significant differences between the sampling sites (Figure 4-6), the percentage of phagocytosing neutrophils (Figure 4-7) and their phagocytic capacity was significantly different between the venous and capillary samples (Figure 4-8).

Research has shown that when travelling from major vessels to vessels of smaller diameter, as from arterioles to capillaries then onto post-capillary venules, leucocytes are subject to conformational changes (Sundd et al., 2013). When passing through smaller vessels with lower diameters, leucocytes do not deform as much as erythrocytes (Chien et al., 1987). This is supported by the lack of reduction in RBC velocity while leucocytes may become almost stationary when travelling from a 9μm diameter to a 5μm capillary (Oertel, 2010). Additionally, the decreased vascular diameter promotes collisions of erythrocytes and leucocytes, leading to margination of the latter to the vascular wall (Sundd et al., 2013). Such physiological margination and decrease in travelling velocity allows for increased interaction between neutrophilic surface receptors, such as low-affinity β2-integrins, and epithelial cells (Anderson, Hotchin, & Nash, 2000). In contact with the vascular epithelium, neutrophils project pseudopods and if not stimulated, they retract the pseudopod, return to spherical shape and resume rolling adhesion as fluid shear force created from the change in vessel diameter acts on it (Fukuda, Yasu, Predescu, & Schmid-Schonbein, 2000; Mitchell, Lin, & King, 2014). Pseudopod projection and adhesion have been associated with expression of adhesion molecules, such as CD11b/CD18 (CR3) (Anderson, 1995), and the continuous activation and deactivation of these molecules enables neutrophilic physiological rolling (Sheikh & Nash, 1996). Until recently, it was thought that only chemotactical stimuli were responsible for the neutrophilic activities described above. However, the mechanotransduction mechanism described has been suggested to regulate pseudopod projection due to physiological, shear-induced, conformational changes and cleavage of CD18 (Shin, Simon, & Schmid-Schonbein, 2008).

Data has also established that haemodynamic conditions vary between vascular regions, imposing forces on each cell such as circumferential stress and shear stress (Papaioannou & Stefanadis, 2005). As shear force has been related to internal diameter of the vessel, it is safe to assume that in the capillary samples obtained, where cells were confined to diameters varying from 4-6μm in arterial capillaries to 10-15μm in post capillary venules (Braverman, 2000), shear force was greater than when cells were obtained from ante-cubital veins, where diameters vary from 1.3-2.9mm (Baptista-Silva, Dias, Cricenti, & Burihan, 2003). Such mechanical stimulus has the ability to remodel the neutrophil cytoskeleton as well as induce cell activation through change in surface receptors, particularly adhesion molecules (Yap & Kamm, 2005). The transient change in expression of CD11b/CD18 could provide insight into the difference in expression of

these receptors observed between the venous and capillary sampling sites. Perhaps once past the mechanical and chemical stimuli in the capillary beds, neutrophils return to an inactivated state and flow freely through venous vascularity. As the findings of the present study support this hypothesis, it becomes apparent that blood collection location must be regarded as an important factor in assays measuring antigen expression in leucocyte subpopulations.

Furthermore, the vasodilatation techniques used in this study may slow flow rate, allowing a "loose and somewhat transient" adhesion (tethering) resulting in rolling of neutrophils along the epithelium (Witko-Sarsat, Rieu, Descamps-Latscha, Lesavre, & Halbwachs-Mecarelli, 2000). The consistency of increased expression of adhesion molecules in capillary-drawn samples (Figure 4-5) may also suggest that the routinely used methods adopted in this research to increase blood flow to these sampling sites, either via action of nonivamide and butoxyethyl nicotinate containing cream - Finalgon® (Zavorsky (Beneke & Alkhatib, 2015; Schommer et al., 2012; Zavorsky, Lands, Schneider, & Carli, 2005) or submersion of limb in hot water, may have contributed to neutrophil activation. Nonivamide has been described to, when applied to the skin, induce release of vasodilative polypeptides such as calcitonin-gene-related peptide (CGRP) and substance P (Stucker, Reuther, Hoffmann, Aicher, & Altmeyer, 1999). CGRP has been shown to promote accumulation of inflammatory cells in areas of inflammation and to enhance neutrophilic adhesion to the endothelium in vitro (Hartung & Toyka, 1989; Zimmerman, Anderson, & Granger, 1992). The later study however, did not associate this neuro-peptide induced neutrophilic adhesion to CD11/CD18, L-selectin, E-selectin, or ICAM-1. Substance P, however, has been linked with priming of mice neutrophils for chemotactic response through the augmentation of CD11b receptors in its surface membrane (Sun, Ramnath, & Bhatia, 2007). Additionally, research has shown a greater expression of CR3 receptors incubated at 37ºC, compared to 4ºC incubation (Mobberley-Schuman & Weiss, 2005). Furthermore, capillary blood collection involved greater tissue trauma compared to venipuncture, possibly increasing the recruitment of activated neutrophils to the area (Hoang et al., 2013).

This study also hypothesised that the function of granulocytes collected from the three different sampling sites would yield statistically different results. The main finding of this study did support such hypothesis, as MANOVA (Pillai's trace) results showed significant differences in phagocytic capacity of granulocytes according to their vascular

location at the point of collection (Figure 4-8). The percentage of neutrophils that underwent phagocytosis was significantly different from vein to finger ($p=0.025$) (Figure 4-7). Most significant was the reduced number of ingested *E.coli*, measured as median fluorescence intensity, between the samples obtained from the vein and both capillary sites (Figure 4-8).

Clearance of pathogens via the process of phagocytosis is dependent on both the concentration of neutrophils and the appropriate functioning of all their killing capacities (Li, Karlin, Loike, & Silverstein, 2004). Since neutrophil concentration between the three different sampling sites did not differ significantly (Figure 4-2), neutrophil supply does not appear to be the source of altered phagocytic capacity in capillary samples.

Abnormal phagocytosis has been attributed to a failure in the opsonisation process or defect in the ingestion capability of the phagocyte (Robinson, Carter, & Narayanan, 1997). Even though phagocytic cells have the ability to engulf both opsonized and non-opsonized particles, phagocytosis is enhanced by the opsonisation process (Lee et al., 2003). The importance of opsonic phagocytosis may be demonstrated by the increased susceptibility to infection suffered by patients who lack components of opsonic systems found in serum, such as immunoglobulin G (IgG) and the complement system (Peiser and Gordon, 2001). The main opsonin receptors in neutrophils are Fc receptors (FcγRIIA (CD32), FcγRIIIb (CD16)) and β2 integrin (CR3, MAC1 (CD11b/CD18)). Decreased expression of CD16 antigen observed in elderly patients has been correlated with attenuated Fc mediated phagocytosis (Butcher, Chahal, Nayak, Sinclair, Henriquez, Sapey, O'Mahony, et al., 2001). However, such molecular event was not found in this study as there was no significant difference in CD16 expression observed between the three sampling sites. Previous research has highlighted the crucial role of serum, particularly complement opsonisation, for efficient phagocytosis of *E.coli*, where unopsonised *E.coli* were not significantly up-taken by neutrophils when compared to opsonised bacteria (Gordon, Rice, & McDonald, 1989; Nishimura et al., 2001). Research has demonstrated a more rapid inactivation of complement factor C3 in serum from capillary samples when compared to serum obtained from a venous sample (Bjorksten, 1973). Such observation could explain the decrease in the number of bacteria ingested by granulocytes from both fingertip and earlobe when compared to those obtained from venous sample. It is possible to suspect then that extracellular factors, such as complement fragment level, may cause the discrepancies observed between venous and capillary

samples, particularly seeing that the expression of CD11b/CD18 molecules, as previously discussed, showed significant increase in capillary sites when compared to venous site (Figure 4-5). The fact that there was no statistical difference (*p=1.00*) in phagocytic capacity between the two capillary samples (finger and earlobe) strengthens this argument (Figure 4-7).

Questions arise since the increased expression of CD11b/CD18 has been correlated with increased phagocytic capacity (Hofman et al., 2000), contrary to the findings in this study. Both fMLP stimulation and cultured intestinal epithelial transmigration of neutrophils increased CD11b/CD18 expression and phagocytic capacity according to Hofman et al. (2000). Even though antigen expression quantification in this study did not make use of any neutrophilic stimulation, the association of increased antigen expression and capacity of these cells to phagocytose more bacteria can still be suggested. Furthermore, occupancy or blockage of CD11b/CD18 has been shown to inhibit binding of not only iC3b opsonised particles but of bacteria such as *E.coli* to neutrophils (Wright & Jong, 1986). While CD11b/CD18 recognises bacterial lipopolysaccharides (Wright & Jong, 1986), the rate in which it uptakes the microorganism, compared to an iC3b coated one is not yet fully described. Gordon et al. (1989) observed increased expression of CD11b/CD18 incubated with opsonised *E.coli* after 30min compared to unopsonised *E.coli*. The difference in CD11b/CD18 kinetics between opsonised and unopsonised *E.coli* was only insignificant after 60 minutes incubation (Gordon et al., 1989). Thus, incubation time of 10 minutes in this study might not have been sufficient to allow phagocytosis of unopsonised *E.coli* via lipopolysaccharide recognition.

Another noteworthy physiological phenomenon that occurs with the decrease in vascular diameter is the formation of platelet-neutrophil complexes (PNC). The previously mentioned displacement of erythrocytes to the central core of the blood vessel pushes platelets and leucocytes towards the vascular wall, allowing increased interaction between them. PNCs have been reported to constitute to approximately 25% of circulating polymorphonuclear leucocytes (Peters, Heyderman, Hatch, & Klein, 1997). PNCs show increased activation through increased expression of adhesion receptors CD11b/CD18 and increased phagocytic capacity (Peters et al., 1999). Such observations however conflict with our findings since even though capillary samples showed greater expression of CD11b/CD18 their phagocytic capacity was less than that observed in neutrophils obtained from the venous blood sample (Figure 4-5 and Figure 4-8,

respectively). Further analysis could be performed by adding platelet-marker CD42b to identify specific neutrophilic capacity of PNCs in different sampling sites.

Conclusion

With increased attention being paid to immunological responses to the physical stress imposed by exercise, the present study demonstrates that valuable information regarding immune cell population distribution as well as specific immune functions may be attained utilizing a minimal amount of blood. The present study has demonstrated that concentrations of erythrocytes and leucocyte subpopulations (lymphocytes, monocytes, eosinophil, basophils and neutrophils) do not vary significantly between capillary and venous blood sampling sites, supporting the interchangeability of sampling sites for these variables. However, it has been shown that antigenic expression and function of leucocytes, particularly neutrophils, do vary according to their vascular location at the time of blood draw. The clinical applications of such conclusions however, must still be demonstrated.

As leucocyte populations have been shown to respond to both biochemical and biomechanical stimuli happening inside the vascular system, sampling site must be taken into account when comparing results between studies. The present study demonstrates that although phagocytic function of neutrophils has been shown to be altered depending on the cells' vascular location, it does not exclude the usage of the micro-sampling method in sports setting. While results do indicate the need for further research to establish reference ranges for the parameters mentioned above based on sampling site, provided that fidelity to sampling site is kept, changes brought about through exercise may be seen in any sampling site.

Despite not being within the bounds of this study, future research should aim to address the questions raised here. As the present study obtained resting samples from healthy individuals, further research will aim to observe if such variations occur when the immune system is under stress. For that, exercise seems to be an ideal model to allow further investigation into the differences between sampling sites as it not only influences blood distribution, but also sets the immune system under stress. Furthermore, in light of the relationship between complement fragments and their capacity to opsonise microorganisms for efficient phagocytosis, further research should consider the availability of complement (i.e. concentration) in serum in different vascular regions.

Also, further studies could aim to quantify antigen expression with and without stimulation (such as chemoattractants) and phagocytic capacity of the stimulated and unstimulated samples on a venous and capillary blood sample.

Chapter 5

Effects of exercise and training on immunological and iron related parameters in elite female kayak athletes during a period of intensified training

Introduction

Throughout a training year, elite athletes are commonly challenged with periods of intensified training. This strategy evokes greater physiological adaptations such as increased maximum strength, peak power and velocity when compared to low-intensity training periods (DeWeese, Hornsby, Stone, & Stone, 2015a, 2015b; Kraemer & Ratamess, 2004). In this training phase athletes are often required to perform work at approximately 85 to 110% of race pace, corresponding to intensities above the second ventilatory threshold (Guellich, Seiler, & Emrich, 2009). If not carefully prescribed, the demands imposed by the increased load may increase sympathetic stress and induce a state of "over-reaching", in which performance is decreased (Halson & Jeukendrup, 2004). Concomitantly, the increased stress may come to affect other physiological processes such as immunity and iron regulation (Magazanik et al., 1988).

Exercise-induced immune modulation is known to be dependent on specific characteristics of training such as duration and intensity. The effects of intensified bouts of exercise on the immune system have been widely reported in both adaptive and innate immune branches (Pyne, 1994). Immediately post-exercise an overall transient increase in blood leucocyte concentration is observed, mainly through increase in concentration of granulocytes, which usually make up 60-80% of circulating leucocytes. Gabriel and Kinderman (1997) demonstrated post-exercise neutrophilia is more pronounced in exercises of long duration (>1.5 hours) rather than HI. Lymphocytes (particularly the $CD3^+$ subsets) show an increase during exercise and immediately post-exercise, with $CD3^+CD8^+$ showing a greater relative increase in concentration (Gleeson & Bishop, 2005), but declining quickly and reaching below pre-exercise values one hour into recovery. Post-exercise distribution of $CD19^+$ lymphocytes in the circulation has been described in literature as 'decreased' (Morgado et al., 2014), 'unchanged' (Natale et al., 2003; Shek, Sabiston, Buguet, & Radomski, 1995) and 'increased' (Nielsen, Secher, Christensen, & Pedersen, 1996). Exercise intensity dictates the magnitude and duration of NK lymphocyte ($CD3^-CD56^+$) mobilization (Gabriel, Urhausen, & Kindermann, 1992), with reports of an increase of over 500% from pre-exercise values (Gabriel & Kindermann, 1997), contrary to the previously described lymphocyte sub-populations (Gleeson & Bishop, 2005). These changes observed in leucocyte sub-populations after a single bout of exercise have been attributed to the increase in apoptosis (via increase in ROS, glucocorticoids and/or Fas ligand) (Krüger & Mooren, 2014) and increase in the

concentration of catecholamines (mainly adrenaline and noradrenaline), cortisol, cytokines (including GM-CSF, IL-6, IL-1, IL-17, IL-23 (Sugama, Suzuki, Yoshitani, Shiraishi, & Kometani, 2012) and acute phase proteins release during exercise that promote leucocyte migration from bone marrow (or marginated pools in organs such as spleen, liver and lung) (Hogg & Doerschuk, 1995; von Vietinghoff & Ley, 2008), to circulation and onto tissue (Northoff, Weinstock, & Berg, 1994). This dynamic redistribution of leucocyte sub-population in peripheral circulation may take up to 24 hours to return to baseline values (Robson, Blannin, Walsh, Castell, & Gleeson, 1999).

Periods of intensified training, where exercise intensity and training frequency are increased, may delay the return of the previously described acute changes in leucocyte concentration to baseline values. Five days of intensified training has been shown to decrease circulating concentration of $CD3^{+}CD4^{+}$, $CD3^{+}CD8^{+}$ and $CD3^{-}CD19^{+}$ lymphocytes from pre-camp baseline values in male soccer players (Malm, Ekblom, & Ekblom, 2004). Mochida et al. (2007) compared circulating leucocyte concentration changes in female collegiate judoists pre- and post-exercise following a 64-day training period and an intensified 6-day training period. Prior to the habitual 64-day training period, a 67% increase in concentration of circulating neutrophils was observed, while after the training camp, only a 34% increase in circulating neutrophils was observed. While a significant increase in neutrophil to leucocyte ratio was observed after both training modes, the percent change in this ratio from pre- to post-exercise before the camp was 32% while after the camp was only 7% (Mochida et al., 2007). After four weeks of intensified training, male triathletes exhibited a lower circulating neutrophil concentration compared to the group that maintained normal training (Coutts, Wallace, & Slattery, 2007). The functional capacity of these leucocyte sub-populations, however, is not necessarily related to changes in concentration (Smith, 1997).

While activation of neutrophils, evidenced by changes in their phagocytic, oxidative capacity and degranulation functions, has been described after a single bout of exercise (Gray et al., 1993; Peake, 2002; Pyne, Smith, et al., 2000; Robson, Blannin, Walsh, Castell, et al., 1999), few have considered multiple training sessions per day, as it is routinely scheduled for elite athletes in intensified training periods (see supplement 1 - Table A). Prasad et al. (1991) has demonstrated that pre-stimulated neutrophils have reduced capacity of both production and release of ROS. Even though this research group demonstrated a recovery in ROS production (measured through chemilumenscence) after

2 hours it is not clear if multiple stimulations (i.e. multiple training sessions per day) would provide similar results. Introduction of interval training sessions in a 2-week training period has been shown to significantly decrease neutrophilic degranulation in elite male triathletes (Robson, Blannin, Walsh, Bishop, & Gleeson, 1999). Elastase (a product of degranulation) released per neutrophil decreased after a 2-week HI training period was introduced amidst a 4-week training camp (Robson-Ansley (Robson-Ansley, Blannin, & Gleeson, 2007). The capacity to produce an oxidative burst, a vital anti-bactericidal function of neutrophils, showed significant decrement after only one week of intensified training in cyclists (Lancaster et al., 2003). Nieman et al. (2014) showed that, the insertion of a 3-day intense training cycle in the 5th week of a 12 week period, caused decrements in neutrophilic phagocytic and oxidative burst capacity. These functions remained decreased after 38 hours post-exercise by 27% and 12%, respectively. Interestingly, this same group did not find any significant difference between sporting disciplines, highlighting that in either cycling or running, the intensity of the exercise performed dictated the decrement in neutrophil function, rather than the mode of exercise undertaken (Nieman et al. 2014). Even though the clinical significance of such variations is still to be elucidated, the significant perturbation of the immune system and the increased susceptibility to opportunistic infections induced by the previously stated stimuli are well established (Gleeson, 2006).

While cellular immunity is highly efficient in exterminating pathogens, other physiological mechanisms are activated once inflammation and/or infection are perceived. One of the strategies is pathogen starvation via iron sequestration as part of the APR. This mechanism is governed by increased circulation of iron-regulating hormone, hepcidin, in response to increased concentrations of the pro-inflammatory cytokine IL-6 (Kemna, Pickkers, Nemeth, van der Hoeven, & Swinkels, 2005; Nemeth et al., 2004; Wrighting & Andrews, 2006). Acute changes in iron parameters have been observed after both moderate and HI single training sessions, indicating that exercise is a sufficient stimulus to perturb iron homeostasis (Cordova Martinez & Escanero, 1992; Newlin et al., 2012; Roberts & Smith, 1990; Skarpanska-Stejnborn, Basta, Trzeciak, & Szczesniak-Pilaczynska, 2015). Exercise intensity impacts circulating iron parameters, as research showed that while incremental running (Bruce protocol) caused a 37% decrease in serum iron, running at 50% HR for 30 minutes at constant speed only caused a 17.4% decline in serum iron levels in national-level female taekwondoists (Rahmani-Nia, Rahnama, & Masoumi, 2007). This same group also noted a significant increase in sTfR concentration

post-incremental running, signalling the increased cellular requirement for iron acquisition. Further, exercise-induced fluctuations in iron parameters seem to be more pronounced in trained subjects when compared to their untrained counterparts (Haymes & Spillman, 1989; Schumacher, Schmid, Grathwohl, et al., 2002).

Recently, attention has been paid to the requirements for iron that may arise during this period of iron sequestration. It is well established that leucocyte populations, such as lymphocytes, require iron for proliferation, activation and DNA synthesis (Breuer, Epsztejn, & Cabantchik, 1995; Neckers & Cossman, 1983). Using transferrin receptor (CD71) expression in the cellular membrane Broadbent (2011) identified greater $CD4^+$ lymphocyte requirement for iron in male triathletes throughout an endurance training year through flow cytometric identification of a double positive lymphocyte population ($CD4^+CD71^+$) in periods of increased training volume and intensity preceding competition. These findings were interpreted as having an inverse relationship with intracellular iron stores as CD71 expression provides an index of iron requirement of an individual cell. According to the author, this suggests that the increased lymphocyte proliferation could be correlated with a decrease risk of infection, as the only two URTI cases reported occurred in the same month in which the lowest percentage of $CD4^+CD71^+$ lymphocytes was recorded (Broadbent, 2011). Little is known about iron requirements in other leucocyte populations, assessed via the expression of CD71, particularly concerning exercise.

The aim of this study was to identify variations of immune function and iron status in elite female kayak athletes during a period of intense training. To do so, acute alterations were analysed measuring variations from pre- to post-training on each testing day as well as a comparison between pre- and post-training samples throughout the training camp. These investigations will quantify the impact of a period of increased training load on the distribution and function of specific leucocyte subsets, as well as iron requirements in elite female kayak athletes.

Methodology

Participants

World-class female kayak athletes (n=7, age=26±3.4 years, mass=73.2±5.6kg, $\sum 7_{skinfold}$= 76.2+15.6mm, $\dot{V}O_{2peak}$=52.0+3.3 mL/kg/min) participated in this study. This select group of athletes is comprised of two medallists at World Championships, two ranked within the top nine in World Championships, three are medallists at the Under 23 World Championships and a member of the K4 which placed 11th in World Championship. These athletes were all national medallists and pre-selected to take part in the training camps from which the Australian Olympic Kayak team would be selected.

Training

The targeted intensity-focused camp was held in April 2015, 17 weeks before the 2015 International Canoe Federation Canoe Sprint and Para-canoe World Championships held in Milan, Italy- a Rio 2016 Olympics qualifying event. The 10-day intensified training period in this study consisted of three daily sessions. The initial session (AM_1) and the second morning session (AM_2) were both "on-water" sessions while the third session (PM) entailed strength training followed, on occasion, by a paddling session. Training was divided into eight training zones according to Bullock et al. (2013), detailed in the methodology section of this thesis (Methodology - Table 3-4). In the AM_1 session athletes trained in both T2 and T5 zones, coming up to T7 and T8 on force testing and time-trial days, respectively. The AM_2 session usually involved 'mini-max' tests (i.e. 750m 'on-water' step test – see supplement I - Table A and B) where athletes accumulated kilometres in the T6 zone. Finally, the PM session was held at the gymnasium with specific individualized strength programmes and was complemented by either 30 minutes of cross training or 15 second bouts of on-water HI, high resistance interval training at T8 zone. All training protocols and scheduling were developed and supervised by the women's senior kayak coaches and AIS physiologists. A detailed training schedule and results from the on-water 750m step test (performed on testing day 1 under AIS senior physiologist's supervision) are provided in the supplement I (Table A and B).

The daily training loads throughout the entirety of the training camp were analysed based on the athlete's daily input of session RPE onto the on-line assessment method previously

established by AIS. Session RPE was based on the Borg Scale (6-20) where increments are linear, reflecting the intensity of the stimulus and physiological responses to it (i.e. heart rate and $\dot{V}O_2$). Training load was quantified based on the product of session RPE and session duration (Foster et al., 2001), recently validated for kayak athletes (Oliveira-Borges et al., 2014). After every training session athletes recorded a RPE through the on-line assessment method previously established by AIS. A seven-day rolling average of the training loads was considered as cumulative training load.

Blood collection

On four non-consecutive days capillary blood was collected from the athlete's earlobe after 5-10 minutes of vasodilative stimulation with Finalgon® cream (Boehringer Ingelheim, Germany) into heparinised capillary tubes (300µL) containing serum separator gel (Kabe Labortecknik, Germany). Capillary samples were collected between 05:00-06:30 hours prior to AM_1 ('pre-training') and at the end of the PM training session ('post-training'), between 15:00-16:30 hours. On the first and last capillary collection day, a third sample was collected immediately after the AM_2 session (Table 5-1).

Table 5-1 – HI camp schedule

Date	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**1**
	T	**W**	**Th**	**F**	**S**	**Su**	**M**	**T**	**W**	**Th**	**F**
Gold Coast Training Camp											
Athlete Arrival/Departure											
Capillary samples (Immune parameters)		TDay 1		TDay 2			TDay 3		TDay 4		
Capillary samples (Iron parameters)											

Schedule of training camp held in April 2015 at AIS Gold Coast training centre. Testing days (TDay) will be later referred to in-text.

Blood samples were immediately transported to the laboratory where analysis of leucocyte phenotypes through monoclonal antibody labelling, neutrophilic phagocytosis of FITC labelled *E.coli* and PMA stimulated oxidative burst function were independently performed (described in the Methodology chapter (3) of this thesis). Throughout all training days the iron requirement by leucocytes was analysed through cell surface expression of transferrin receptor (CD71). Remaining capillary blood (~ 150µL) was centrifuged and serum was stored at -80°C for analysis of iron parameters. Serum iron, transferrin, sTfR and UIBC were analysed by the COBAS Biochemical Analyser 400 plus

(Roche Diagnostics, Switzerland). Capillary samples were diluted 1:3 reaching total volume of 100µL required for analysis. Serum hepcidin concentration was performed in once-only thawed samples through commercially available ELISA kit (Quantikine® ELISA DHP250 – R&D Systems), abiding by manufacture's manual. Samples were read in 450nm with wavelength correction at 570nm. Samples were analysed in duplicate and mean intra-assay CV was 6.7%.

Statistical Analysis

Acute responses to the training day, from pre AM_1 session to post PM session, were analysed through paired samples t-test after data met the required assumptions. Where data failed normality assumptions, a Wilcox Signed Rank test was performed. Significance was set at $\alpha \leq 0.05/4$ to correct for the four testing days analysed together (Bonferroni adjustment). Also using the acute measures (pre AM_1 session to post PM session), an exploratory Pearson's product-moment correlation was made to determine the existence, if any, of a linear association between the variables analysed. Calculation of effect size (ES) was performed in every paired sample *t*-test according to Cohen's *d*, where difference between means is divided by the half the sum of the standard deviations (Cohen, 1988). Effect size was considered "small" if $d \leq 0.2$, "medium" if $d = 0.5$ and "large" if $d \geq 0.8$.

Combined analysis of the samples obtained pre AM_1 session from each training day provided information on the daily variation of the parameters analysed. Repeated measures linear mixed model analysed the impact of each training day to phenotypical distribution of leucocyte sub-populations and the neutrophilic functions described above. Finally, a repeated measures linear mixed model analysis was used to examine the effect of changes in the expression of antigens on the granulocyte's surface on the capacity of these leucocytes to ingest fluorescently labelled *E.coli*.

Selecting a repeated measures mixed model for statistical analysis preserved the independence between the athletes while taking into account the correlation between the repeated measures (training day). Mixed modelling, used here and in the subsequent chapters, accounted for the small sample size (Bell et al., 2010) and for possible missing data points (particularly from AMS data), reviewed in Ibrahim and Molenberghs (2009). All models were initially fitted for fixed effects based on the smallest Akaike's

Information Criterion (AIC) and lowest number of parameters, using an estimation method of maximum likelihood. Once fixed effects were determined, different covariance structures for repeated and random effects using a restricted maximum likelihood estimation criterion were compared. This model was selected based on smallest values for AIC for both repeated and random covariance structures. As days were repeated, and the correlation between of the outcome was expected to decrease as times points got further apart, the best covariance structure for the repeated measures was auto-regressive 1 (Beaumont, 2012). Taking into account each athlete's baseline and slope, a random intercept and slope (day) was applied using a scale identity covariance structure. The model's suitability was determined by checking normality, multicollinearity and homoscedasticity of the residuals. Then, model-predicted values were plotted against residuals and measured values. Residuals were also explored for outliers.

Results

Training Load

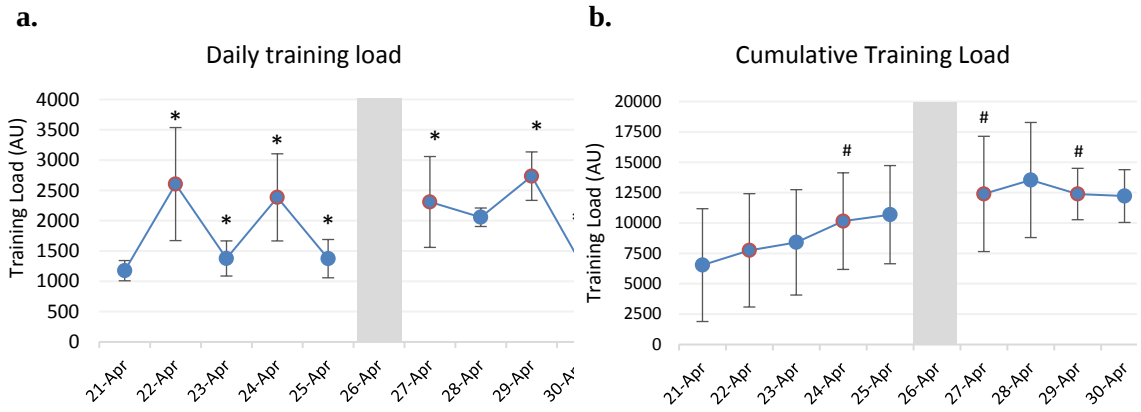
The HI camp accumulated a total of 122 km over 632 minutes of training throughout a 10-day period. The allocation of time and distances per training zone is detailed in table 5-2.

Table 5-2 – Training distance and time per training zone

	T1	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**	**T8**
Total Distance (km)	29.0	44.5	9.5	19.2	8.4	10.2	0.9	0.2
Total Time (minutes)	174.0	244.7	47.5	86.4	33.6	42.0	3.2	0.7

No significant differences between training loads on the testing days were found. However, there were significant differences between training loads throughout the camp. Figure 5-1a shows variability in training load per day of the training camp. Further, calculated cumulative training load, which considered training load from previous training days, showed significant difference from the beginning to the end of the training camp (Figure 5-1b). The constant increase in cumulative load (Figure 5-1b) highlighted significant differences from TDay 1 to TDay 2 (p=0.025), TDay 3 (p<0.001) and TDay 4 (p<0.001).

Figure 5-1 – Daily and cumulative training load per day of camp



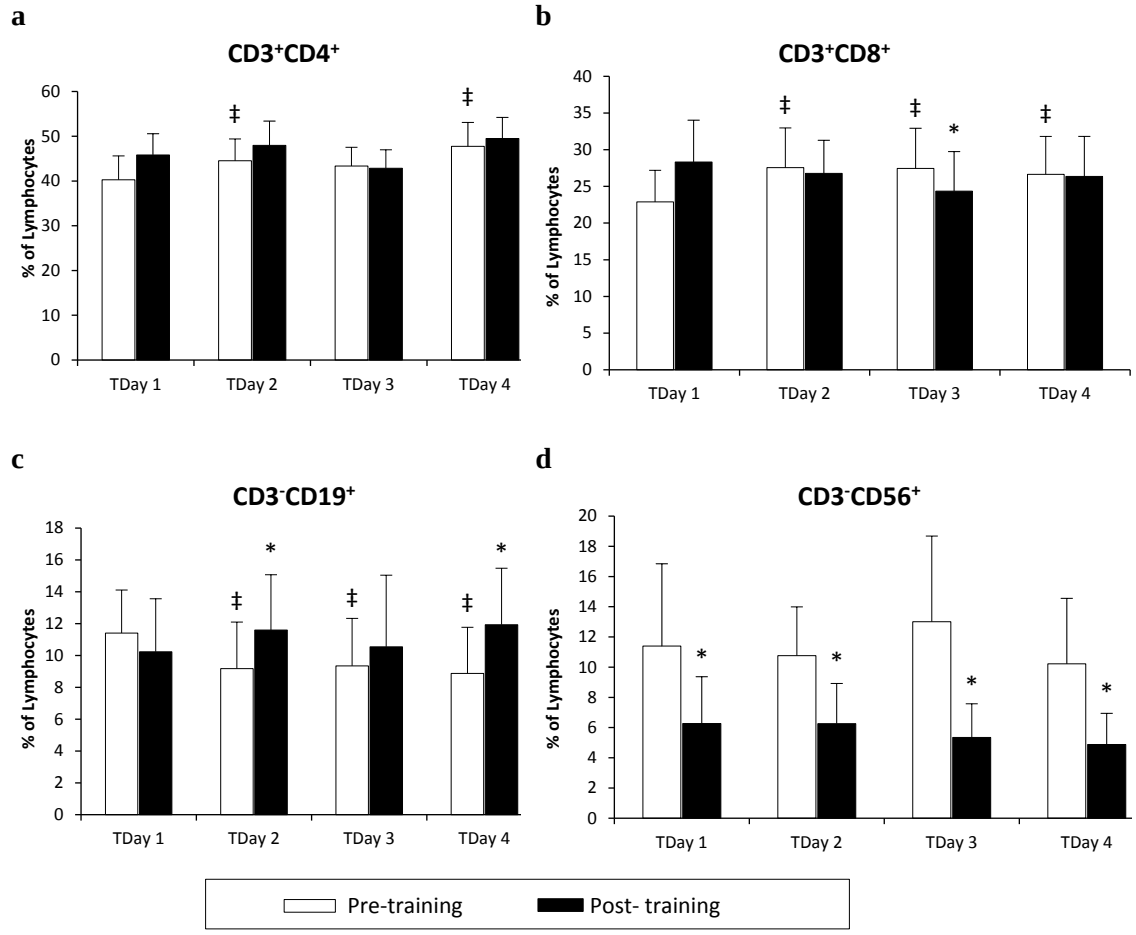
Solid bars represent mean values and error bars represent SD.
* Training load was significantly different (p<0.05) to that measured on previous training day.
# Cumulative training load significantly different (p<0.05) to TDay 1 (22-Apr).
Testing days are marked in red. 26-April is highlighted as a full resting day, hence no training load.

Acute Changes

Leucocyte phenotypical distribution and expression

Specific lymphocyte sub-populations (expressed as a percentage of total lymphocytes) fluctuated from pre- to post-training samples (Figure 5-2 a-d). $CD3^{+}CD8^{+}$ lymphocyte concentration was increased in the post-training sample in TDay 1, but declined from pre-sample in every other training day, reaching significance on TDay 3 (p=0.009). The percentage of B lymphocytes ($CD3^{-}CD19^{+}$) in the sample collected after training was increased from pre-training values on TDay 2 (p=0.009) and TDay 4 (p=0.004). Post-training samples showed a consistent trend towards a decline in the percentage of circulating $CD3^{-}CD56^{+}$ lymphocytes in every testing day, with decrements of over 40% from daily baseline values (Figure 5-2d).

Figure 5-2 – Distribution of circulating lymphocyte subsets pre- and post-training



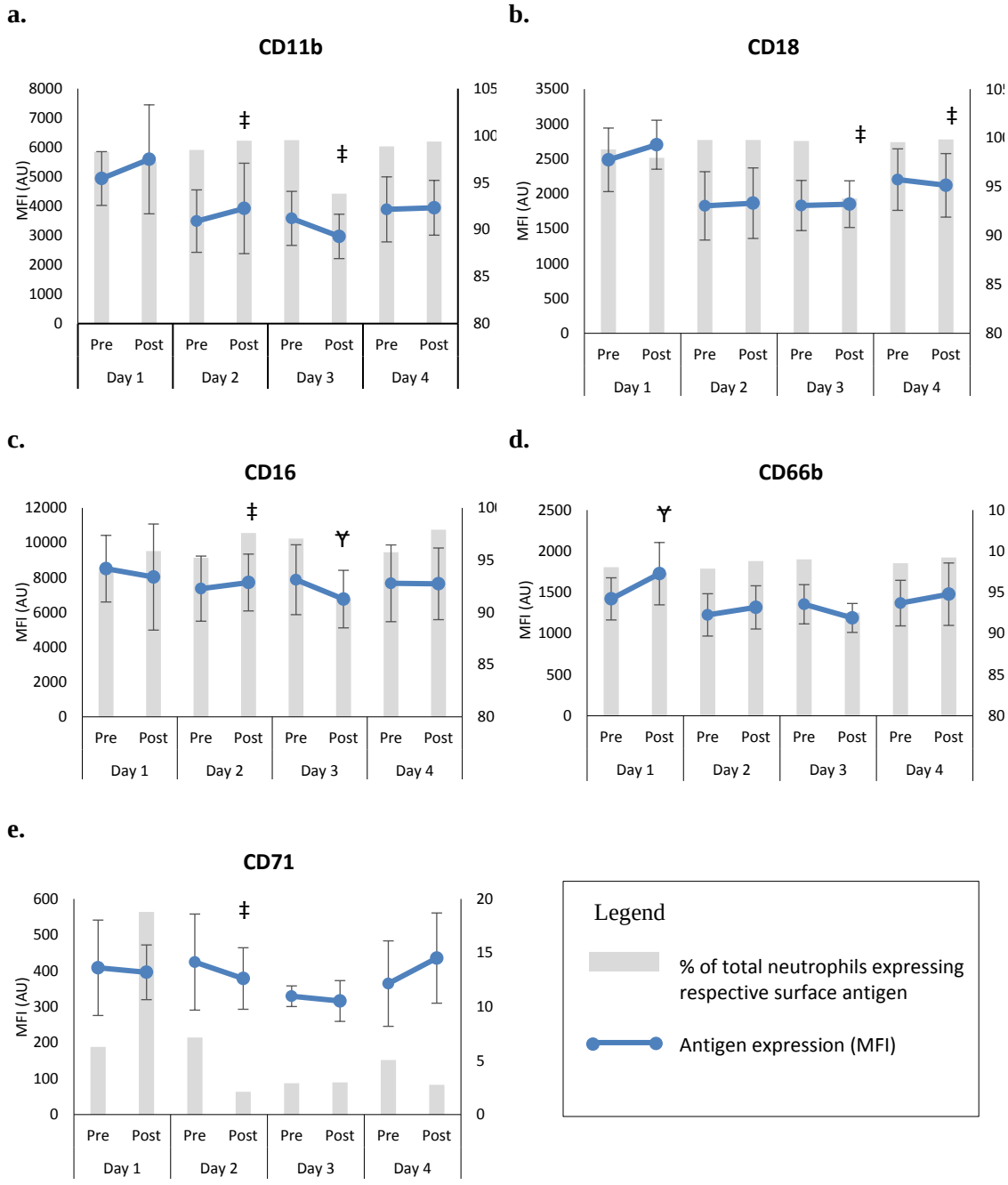
Solid bars represent mean values and error bars represent SD.
* Significant difference from pre-training values ($p < 0.01$)
‡ Significant difference in pre-training values compared to baseline (pre TDay 1) ($p < 0.05$)

Acute changes, measured per day, from pre- to post-training, did not show any significant changes in granulocytic expression of CD11b and CD18, while the expression of CD66b, CD16 and CD71 varied throughout the camp (Figure 5-3a-e). A significant (p=0.012) increase in CD66b expression was observed in TDay 1 from pre- (1421±256 AU) to post-training (1727±378 AU) (Figure 5-3d). On TDay 3, a significant decline (p<0.001) was observed in the expression of CD16 from pre- (7875±2000 AU) to post-training samples (6762±1652 AU) (Figure 5-3c).

The percentage of granulocytes expressing the respective markers also varied throughout the camp (Figure 5-3 a-e). TDay 3 was marked by significant decrements in the percentage of granulocytes positive for CD11b, CD18, CD66b and CD16. The results observed in TDay 3 were in contrast to the results obtained for TDay 2 and 4 where the

percentage of granulocytes positive for CD16, CD66b, CD11b and CD18 in the post-training sample was increased (TDay 4 only) (Figure 5-3 a-e). The percentage of granulocytes expressing CD71, declined significantly on the next testing day (p=0.005). For further details please see Supplement I- Table C.

Figure 5-3 – Phenotypical expression and distribution of granulocytes pre- and post-training

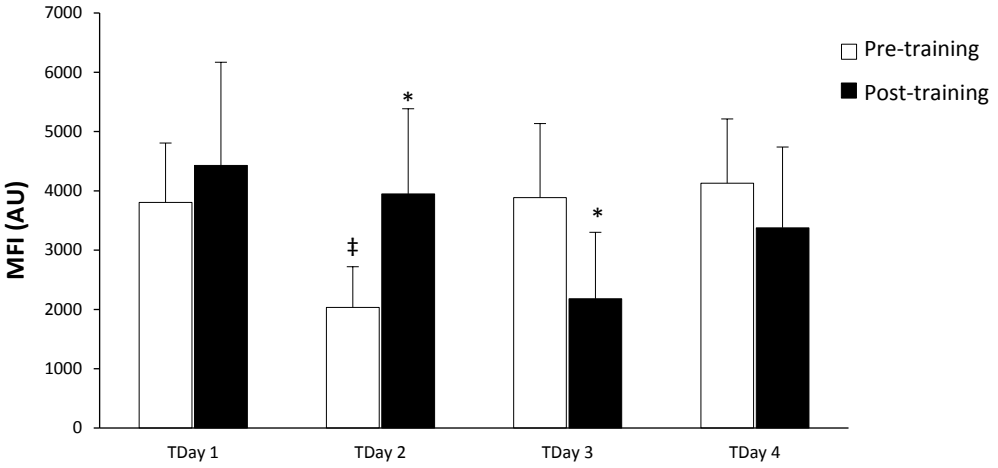


Solid bars represent mean values and error bars represent SD. *df* = 6 for all paired T-tests performed.
‡ Significant at $p< 0.01$ (bar graph) Ɏ Significant at $p< 0.01$ (line graph)

Granulocyte function

A significant increase (p=0.001) in phagocytic capacity (measured by MFI) was observed in the post-training sample of TDay 2 (2785±1277 AU), compared to the pre-training sample (742±432 AU) obtained on the same day (Figure 5-4). On TDay 3 however, there was a significant reduction (p<0.001) in ingested *E.coli* observed in the post-training sample (1174±838 AU) compared to that obtained prior to training (2750±937 AU).

Figure 5-4 – Phagocytosis of *E.coli* by granulocytes



Solid bars represent mean values and error bars represent SD.
* Values differ significantly (p<0.01) between pre- and post-training samples.
‡ Significant difference between pre-samples compared to baseline (pre-sample TDay 1).

The percentage of granulocytes participating in phagocytosis of *E.coli* varied throughout the training camp. An increase from pre- to post-training of 3.4% and of 18.8% in TDay 1 and TDay 2, respectively, were followed by a significant 20% decline in TDay 3 (p=0.008) (Table 5-3).

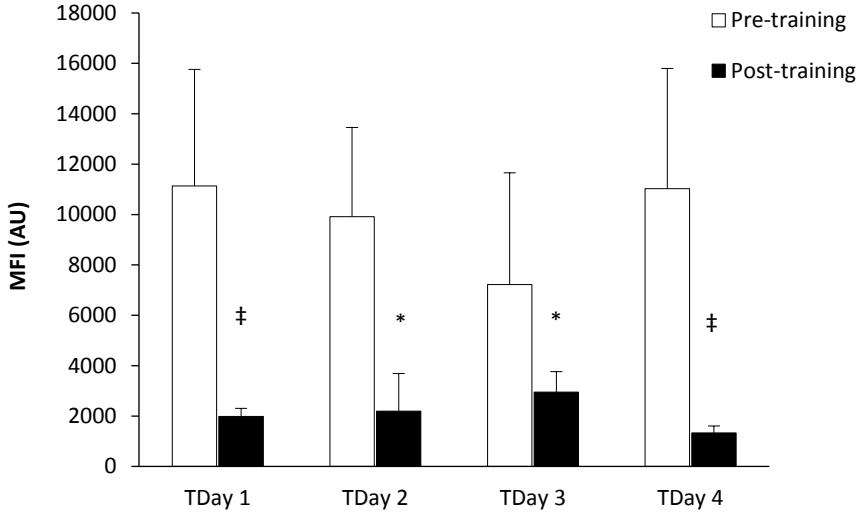
Table 5-3 – Granulocyte phagocytosis

			Mean	SD	% Change	*t*	*p*	ES
Phagocytosis (% of total granulocytes)	TDay1	Pre	94.6	2.2	3.4	-2.845	0.029	1.35
		Post	97.9	2.6		*2.000*	*0.043**	*0.77*
	TDay2	Pre	80.2	17.4	18.8	-2.674	0.037	1.46
		Post	95.2	3.2		*0.000*	*0.018**	*0.89*
	TDay3	Pre	93.9	3.0	-20.1	3.936	0.008‡	-2.11
		Post	75.0	14.9				
	TDay4	Pre	96.2	3.0	-2.7	1.852	0.113	-0.63
		Post	93.6	5.2				

Values in italics – Due to violations of normality a Wilcoxon signed rank test were performed. For these days, columns should read, t = T and Cohen's d = r. *df* = 6 for all paired T-tests performed. * Significant at p<.05 ‡ Significant at p<0.01

H_2O_2 generation through stimulation of oxidative capacity showed a significant decline of over 60% from pre- to post-training samples in every training day (Figure 5-5). The percentage of granulocytes participating in PMA-stimulated ROS formation (measured by H_2O_2 generation) was significantly decreased from pre- to post-training samples (Table 5-4).

Figure 5-5 – Granulocyte stimulated oxidative burst capacity



Solid bars represent mean values and error bars represent SD.
* Significant at $p<.05$ ‡ Significant at $p<0.01$

Table 5-4 – Effects of exercise on granulocytes' stimulated capacity to generate ROS

			Mean	SD	% Change	*t*	*p*	ES
Oxidative Burst (% of total granulocytes)	TDay1	Pre	94.9	3.5	2.6	-1.326	0.233	0.81
		Post	97.2	2.2				
	TDay2	Pre	93.6	4.3	-32.2	2.694	0.036*	-1.82
		Post	63.3	29.1				
	TDay3	Pre	96.9	1.5	-74.1	17.964	<0.001‡	-11.73
		Post	25.1	10.8				
	TDay4	Pre	97.1	2.7	-41.0	3.732	0.01‡	-2.63
		Post	57.1	27.7		*0.00*	*0.018**	*-0.89*

Values in italics – Due to violations of normality Wilcoxon signed rank test was performed. For these days, columns should read, t = T and Cohen's d = r. *df* = 6 for all paired T-tests performed. (*) Significant at $p<$ 0.05 (‡) Significant at $p<0.01$

Iron Studies

While there was a trend to decreases in serum iron concentration and consequently transferrin saturation found in the post-training samples, statistical significance was not reached. Such changes were accompanied by increases in serum transferrin, UIBC and TIBC, again, without reaching statistical significance. At the end of TDay2, the

concentration of serum hepcidin was significantly increased by 78% from the pre-training value (Table 5-5).

Table 5-5 – Iron parameters prior to and at the end of TDay 2

	Pre-training		Post-training		Mean % Change	*t*	*p*	ES
	Mean	SD	Mean	SD				
Serum Iron (μmol/L)	10.5	3.2	9.6	4.6	-7.3	.516	.625	-.24
Transferrin (μmol /L)	30.7	3.7	35.8	9.7	16.7	-1.595	.162	.76
UIBC (μmol/L)	35.0	8.1	42.0	11.6	22.7	-1.780	.125	.72
TIBC (μmol/L)	45.5	7.1	51.6	15.0	14.5	-1.128	.302	.55
TSAT (%)	23.7	7.9	18.3	5.8	-20.8	2.359	.056	-.78
Hepcidin (nM)✧	2.4	0.6	4.2	1.2	78.1	-4.179	.025*	2.06

* Significant at $p<0.05$ $df = 6$ for all paired 2-tailed T-tests performed.
✧ Values were converted from ng/mL to nM, based on 1 ng/mL= 0.358 nM for the predominantly occurring hepcidin-25 (Ganz, 2003b, 2008)

Using the data collected during the second training day, Pearson's two-tailed correlation between the variables depicting iron parameters and neutrophil functions and phenotypes was assessed (Supplement I - Table D). Pre-training samples were analysed separately from post-training samples. Pre-training samples showed a very strong positive correlation between serum iron and CD11b (r=.861, n=7, *p*=.013) and CD18 (r=.800, n=7, *p*=.031) expression. These correlations were not present in post-training samples, with values, although non-significant, showing a trend towards a moderate negative correlation. CD71 showed a very strong positive correlation with UIBC (r=.826, n=7, *p*=0.022) and TIBC (r=.830, n=7, *p*=0.021) and a strong negative correlation with CD16 (r=-.774, n=7, *p*=0.041) were observed in the pre-training sample, but not in the post-training sample. The sample collected at the end of the second training day showed a very strong correlation between CD16 and transferrin (r=.876, n=7, *p*=0.010), UIBC (r=.846, n=7, *p*=0.016) and TIBC (r=.867, n=7, *p*=0.012). Further, there was a strong negative correlation between *E.coli* ingested and TSAT (r=-.783, n=7, *p*=0.037) in the pre-exercise sample but not in the post-training sample. The post-training sample showed a very strong correlation between *E.coli* ingested and expression of CD66b (r=.844, n=7, *p*=0.017) and CD11b (r=.844, n=7, *p*=0.017). There was a very strong positive correlation between expression of CD11b and CD18 in both pre- (r=.984, n=7, *p*<.001) and post-training (r=.954, n=7, *p*=0.001) samples. CD66b was very strongly correlated with expression of CD11b (r=.857, n=7, *p*=0.014) and CD18 (r=.840, n=7, *p*=0.018) in the post-training samples.

Daily Variations

Cell concentration and phenotype expression

Despite showing no significant change from pre- to post-training samples, the percentage of circulating $CD3^+CD4^+$ lymphocytes was increased by 10.5% and 18.6% from baseline on pre-training samples obtained on TDay2 (p=0.020) and 4 (p=0.016), respectively (Figure 5-2a). Contrarily, the $CD3^-CD56^+$ sub-population, which underwent the greatest variation from pre- to post-training, did not show any significant cumulative effects when comparison between pre-training samples per training day was made (Figure 5-2d). $CD3^+CD8^+$ lymphocytes increased by approximately 20% from baseline on pre- training samples on TDay2 (p<0.001) and TDay3 (p=0.001), and, despite a small decline on TDay4, values were still 16% higher than baseline values (p=0.018) (Figure 5-2b). B lymphocytes expressing $CD3^-CD19^+$ demonstrated an opposite trend, declining ~20% from baseline on pre-training TDay2 (p=0.002), TDay3 (p=0.007) and TDay4 (p=0.006) samples (Figure 5-2c).

Samples obtained prior to training differed between days, suggesting a possible effect of the training loads imposed (Table 5-6). At the onset of the second training day, values for adherence-related surface receptors CD11b and CD18 were significantly decreased (p=0.008 and p=0.003, respectively) from baseline values by approximately 30%. Values for expression of surface receptors CD16 and CD66b obtained prior to the training on TDay2 were also significantly reduced (p<0.001 and p=0.010, respectively) by ~15% from baseline values. Neutrophilic CD11b and CD18 expression was still decreased on pre-training samples obtained on TDay3 (p=0.002 and p=0.006, respectively), as was the expression of CD16 (p=0.008). Expression of CD11b showed a tendency to return to baseline values but was still significantly decreased on pre-training TDay4 samples (p=0.034). Contrarily, CD16 expression decreased from the previous testing day, showing even further decline compared to baseline (p=0.003) (Table 5-6). Post-training samples did not show any significant differences between days.

The percentage of circulating granulocytes expressing CD11b, CD16, CD18 and CD71 varied throughout the training camp (Table 5-6). Circulating $CD11b^+$ granulocytes showed a significant increase from baseline (pre-training TDay1) on TDay3. This testing day was also marked by a significant increase in $CD16^+$ granulocytes and a significant decline in $CD71^+$ granulocytes when compared to baseline. The percentage of gated

granulocytes however, showed a declining trend from baseline in every pre-training sample obtained thereafter (Table 5-6).

Table 5-6 – Cumulative effects of training on selected granulocyte phenotypes

		Mean	SD	% Change from Day 1	*p* (compared to TDay 1)	% Change from previous testing day	*p* (compared to previous testing day)
CD11b MedianFI (AU)	TDay1	4940	916				
	TDay2	3489	1063	-29.4	0.008‡	-29.4	0.048
	TDay3	3582	920	-27.5	0.002‡	2.7	1.000
	TDay4	3889	1108	-21.3	0.034	8.6	1.000
CD16 MedianFI (AU)	TDay1	8511	1913				
	TDay2	7363	1871	-13.5	<0.001‡	-13.5	
	TDay3	7875	2007	-7.5	0.008‡	7.0	0.150
	TDay4	7670	2205	-9.9	0.003‡	-2.6	1.000
CD18 MedianFI (AU)	TDay1	2486	456				
	TDay2	1827	490	-26.5	0.003‡	-26.5	
	TDay3	1831	359	-26.3	0.006‡	0.3	1.000
	TDay4	2203	441	-11.4	0.219	20.3	0.411
CD66b MedianFI (AU)	TDay1	1421	256				
	TDay2	1226	257	-13.7	0.01‡	-13.7	0.063
	TDay3	1355	238	-4.6	0.353	10.5	0.425
	TDay4	1370	277	-3.6	0.487	1.1	1.000
CD71 MedianFI (AU)	TDay1	706	251				
	TDay2	553	102	-21.7	0.19	-21.7	1.000
	TDay3	650	132	-7.9	0.461	17.5	1.000
	TDay4	804	206	13.9	0.336	23.7	1.000
CD11b (% of granulocytes)	TDay1	98.3	1.1				
	TDay2	98.5	0.4	0.2	0.531	0.2	1.000
	TDay3	99.5	0.1	1.3	0.002‡	1.1	0.036
	TDay4	98.9	0.5	0.6	0.105	-0.7	0.316
CD16 (% of granulocytes)	TDay1	93.9	3.0				
	TDay2	95.2	1.5	1.4	0.044*	1.4	0.263
	TDay3	97.1	1.8	3.4	<0.001‡	1.9	0.049
	TDay4	95.8	2.4	2.0	0.023*	-1.3	0.274
CD18 (% of granulocytes)	TDay1	98.8	1.5				
	TDay2	99.8	0.2	1.0	0.046*	1.0	0.278
	TDay3	99.7	0.3	0.9	0.053	-0.1	1.000
	TDay4	99.6	0.3	0.7	0.092	-0.1	1.000
CD66b (% of granulocytes)	TDay1	98.1	1.7				
	TDay2	97.9	1.4	-0.2	0.793	-0.2	1.000
	TDay3	99.0	0.6	1.0	0.146	1.1	0.748
	TDay4	98.6	0.8	0.5	0.451	-0.5	1.000
CD71 (% of granulocytes)	TDay1	6.3	2.9				
	TDay2	7.2	4.3	14.0	0.486	14.0	1.000
	TDay3	2.9	2.2	-53.8	0.007‡	-59.5	0.023
	TDay4	5.1	3.3	-19.3	0.34	74.7	0.599
Granulocytes (% of leucocytes)	TDay1	40.9	7.7				
	TDay2	33.1	5.4	-19.1	0.026*	-19.1	0.159
	TDay3	37.4	8.6	-8.7	0.31	12.8	1.000
	TDay4	32.6	10.7	-20.3	0.038*	-12.7	0.932

*Significant at $p<0.05$ ‡ Significant at $p<0.01$

Granulocytic Function

When comparing all pre-training samples, the percentage of granulocytes participating in phagocytosis of *E.coli* did not differ from baseline to subsequent training days. However, *E.coli* phagocytosis, as indicated by MFI, was significantly decreased in samples obtained prior to TDay2 (2032±687 AU) compared to baseline (3805±1002 AU p=0.001), TDay3 (3885±1250 AU p<0.001) and TDay4 (4130±1083 AU p<0.001) (Figure 5-1).

Interaction of granulocytic functional capacity and antigenic expression was modelled considering repeated measures for day. As day of testing became further from baseline, auto-regressive 1 covariance structure was determined for repeated effects of day. The number of *E.coli* ingested, quantified through fluorescence intensity, was considered the outcome, while considering day a factor and each CD expression a covariate. Due to the small sample size, the effect of each surface antigen expression in granulocytic phagocytosis of *E.coli* was modelled separately. Using only samples obtained prior to each training day, linear mixed model found a significant effect of the expression of CD11b (p=0.032) and CD18 (p=0.038) on the phagocytic capacity of granulocytes. Samples obtained at the end of each training day did not show any effects of antigen expression in the number of bacteria ingested by granulocytes.

Despite the significant acute differences from pre- to post-training described above, when analysing all pre-training samples between the testing days, no cumulative effect of exercise could be observed in granulocytic involvement or in its capacity to produce ROS (Figure 5-5).

Discussion

This study examined the effect of one HI training camp on leucocyte sub-populations, granulocytic function, and iron parameters in elite female kayak athletes. Results from this study demonstrate the exercise-induced (acute) phenotypical heterogeneity of circulating lymphocytes and granulocytes and highlighted cumulative effects of exercise on these populations. Granulocytic functional receptors for FCγRIII (CD16) and adhesion (CD11b/CD18) demonstrated decreased expression from TDay1 throughout the HI camp. The latter finding correlated with the decrease in neutrophilic function of

phagocytosis of *E.coli* from TDay1 in the all the remaining resting samples obtained throughout the HI camp. In contrast, the cumulative effects of exercise were not observed in ROS production, despite its acute pre- to post-training variations.

Exercise-induced changes in lymphocyte distribution have been described extensively (Kakanis et al., 2010; Morgado et al., 2014; Natale et al., 2003; Ronsen et al., 2001). Malm et al. (2000) identified an increase in muscle concentration of $CD56^+$ lymphocytes post-exercise. Muscle damage, quantified by biochemical markers (creatine kinase) and inflammatory markers (C-reactive protein and IL-6), has been reported post-exercise, particularly eccentric exercise (Margaritelis et al., 2015). The decline in circulating levels of $CD56^+$ lymphocytes observed in this study (Figure 5-2d), may be rationalised by the proposed participation and regulatory function of NK cells in muscle repair (Malm et al., 2000; Robertson, Grounds, & Papadimitriou, 1992). Similarly to Nielsen et al. (1996), who observed a 2-fold increase in $CD19^+$ lymphocytes after a first bout of maximal exercise and a 5-fold increase after the last (sixth) bout of maximal exercise in elite rowers, this study observed an increase in $CD19^+$ in samples collected post-training (Figure 5-2c). It is important to highlight that Bertouch et al. (1983) and Huang et al. (2015), have demonstrated significant diurnal variation in $CD19^+$ lymphocytes, highlighting their increase in circulating concentration and percentage on samples obtained in the afternoon (from 1500-1800 hours) compared to samples collected at 0800 hours. The diurnal peak observed by the aforementioned authors corresponds to the sampling time of this study. When analysing samples collected at the same time (prior to training – all before 0700 hours), the percentage of $CD19^+$ lymphocytes throughout the training camp showed significant differences compared to baseline (TDay1). It has been established that throughout the recovery period (from 1 hour post exercise) the concentration of this population, as well as all other lymphocyte sub-populations, decreases from pre-exercise values. However, studies have typically shown lymphocyte concentrations have returned to baseline values with 24 hours rest post-exercise (Kakanis et al., 2010), thus the decline in the percentage of circulating $CD19^+$ lymphocytes observed in the daily pre-training values (Figure 5-2c) may stem from the cumulative effect of consecutive HI training days.

Research has shown that exercise intensity is an important factor in determining the phenotypical distribution of circulating granulocytes (Peake et al., 2005; Shephard, 2003). A significant ~5% decline in the percentage of circulating $CD11b^+$, $CD18^+$, $CD16^+$ and $CD66b^+$ granulocytes from pre-training to post-training was observed on the third testing

day (Figure 5-3), suggesting migration of these populations from the circulation into tissues. The observed decline could be explained by the successful infiltration of these populations towards muscle tissue as to repair exercise-induced muscle damage. Malm (2000) demonstrated a 12-fold increase in the concentration of $CD11b^+$ neutrophils in muscle biopsies obtained six hours post-eccentric exercise. Further studies corroborate the notion of exercise-induced neutrophilic infiltration into tissues by showing an increased concentration of $CD16^+$ (Paulsen et al., 2010) and $CD66b^+$ (Vella et al., 2016) granulocytes in muscle tissue immediately after exercise with significant peak three hours of post-exercise.

Granulocytic surface antigen expression is correlated with function- and activation-induced cytoplasmic rearrangements. Up-regulation of CD66b in the neutrophilic plasma membrane, as observed in the post-training samples obtained on TDay1 (Figure 5-3d), has been associated with degranulation of specific granules (location of CD66b intracellular stores) (Skubitz, 1999). Degranulation post-exercise has been reported previously (Gray et al., 1993; Peake, 2004; Robson, Blannin, Walsh, Bishop, et al., 1999). The observed 13% increase in CD11b expression from pre- to post-training on TDay1 further substantiates the suggestion of neutrophilic degranulation post-training (Figure 5-3a). CD11b is expressed sub-cellularly in secretory vesicles, gelatinase and specific granules (Sengeløv, Kjeldsen, Diamond, Springer, & Borregaard, 1993). At the earliest perception of chemoattractive signals, secretory vesicles are mobilized to increase membrane expression of CD11b and ensure firm adhesion between neutrophil and endothelium ICAM-1 (Borregaard, Kjeldsen, Lollike, & Sengeløv, 1995). Non-localized (i.e. not restricted to adhesion site) up-regulation of CD66b on neutrophilic cellular membrane has been reportedly triggered within 60 seconds of neutrophil adhesion (Naucler, Grinstein, Sundler, & Tapper, 2002).

Curiously, contrary to the observed post-exercise increase in neutrophilic CD16 expression in TDay2, CD16 expression in this population was significantly decreased on TDay3 (Figure 5-3c). Such decline in this FcRγIII receptor expression has been previously described post-exercise (Peake, 2004). CD16 is sensitive to cleavage by elastase (Tosi & Berger, 1988). Therefore, if, as mentioned previously, exercise is contributing to neutrophilic degranulation, and consequently release of elastase (Gleeson et al., 1998; Gray et al., 1993; Robson, Blannin, Walsh, Bishop, et al., 1999), it is reasonable to believe that such a mechanism may be at least in-part responsible for

decreased surface expression of CD16. Up-regulation of CD16 from intracellular stores in neutrophils has been shown to occur only once the surface CD16 has been cleaved (Tosi & Zakem, 1992). Given the functional importance of CD16 (i.e. phagocytosis; Cohen, 1994) , it is not surprising that CD16 is consistently expressed on the neutrophilic surface (resting and activated), and the levels of cell surface expression are maintained by recruitment of intracellular stores. Increased concentrations of shed CD16 found freely in serum have been associated with inflammatory conditions (Fleit et al., 1992; Kabutomori, Iwatani, Koh, Fushimi, & Amino, 1993). Not coincidentally, on the same day (TDay3) that CD16 expression was decreased post-exercise, so was the granulocytic ingestion of *E.coli* (Figure 5-4). Butcher et al. (2001) have shown a highly significant correlation between CD16 expression and phagocytic index (r=0.83; p<0.05). As CD16 is shed by elastase, possibly the exercise-induced granulocyte pre-activation (via cytokines) (Peake et al., 2005), or degranulation post-training, diminishes granulocytic capacity to subsequently carry out phagocytosis.

It is important to highlight that the expression of neutrophilic surface antigens analysed in this study post-training did not seem to follow a particular trend. While there was a tendency towards the increase in expression of CD11b, CD18 and CD66b post-training on TDay1 and TDay2, these observations were not noticed on TDay3. Interestingly, all granulocyte surface antigens analysed presented decreased expression in TDay3. Lacking a significant difference in the acute training load between the testing days, the peculiarity of TDay3 lies on the 24 hour rest the athletes had prior to it (Figure 5-1). After the day of rest, values of the expression of CD11b, CD18 and CD66b obtained pre-training were higher than values obtained on the previous day, but were still lower than baseline values (Table 5-6). This shows that consecutive training days with multiple training sessions do take a toll on both the distribution and function of leucocytes, and highlights the importance of rest to resume optimal functional capacity of the immune system. However, based on the inability of the expression of functional-related markers to return completely to baseline values, the kinetics of the response remain to be fully described as one rest-day is likely to be insufficient to completely recover from exercise-induced declines in immune function.

Despite post-training increases in most of the neutrophilic surface receptors analysed, values pre-exercise on the next testing day were significantly decreased. The post-training increase in expression of the surface antigens described marks an activated state (i.e.

degranulation), possibly related to exercise-induced increases in the concentration of pro-inflammatory cytokines (i.e. IL-6, IL-8, G-CSF) and hormones (i.e. cortisol, growth hormone), which although not measured here, are well described in literature to influence neutrophilic recruitment and functions (Mullen, Windsor, Walsh, Fowler, & Sugerman, 1995; Shephard, Rhind, & Shek, 1994; Suzuki et al., 2000). The decrease in neutrophilic surface antigen expression at least 12 hours post-exercise (overnight), could be due to a refractory period neutrophils undergo post-activation. It is this refractory period that has been postulated by other researchers as the window of opportunity for opportunistic pathogens (Gray et al., 1993; Pyne, 1994; Smith & Pyne, 1997). While it is appreciated that testing is not performed on the same neutrophils tested (i.e. same sample), it is plausible that the high physical demand of the HI training maintains increased levels of circulating cytokines or other serum factors for longer periods, which would come to affect circulating neutrophils hours after the cessation of training.

The stimulated capacity of neutrophils to produce H_2O_2 was significantly reduced in every end of day sample obtained (Figure 5-5). This study employed the use of PMA to stimulate, in the presence of intracellular-calcium (Allard, Long, Block, & Zhao, 1999), protein kinase C, which phosphorylates cytosolic proteic members of the NADPH oxidase, promoting its assembly. Through this methodology, surface receptor binding, which would initiate G-protein coupling leading to PIP2 hydrolysis into DAG and IP3 thus enabling release of intracellular calcium from the endoplasmic reticulum, is by-passed. The use of this methodology assures that the decrements in ROS were not surface receptor-mediated, thus restricting possible mechanisms to the distal part of the signalling cascade in which NADPH oxidase assembly and functioning occur. There is evidence that NADPH oxidase assembly is not restricted to the neutrophil's cytoplasmic-membrane, as it also occurs in specific granules, demonstrable by the presence of cytochrome b_{558} in its membrane (Ambruso, Cusack, & Thurman, 2004; Vaissiere et al., 1999). PMA is known to promote translocation of NADPH oxidase cytosolic components $p47^{phox}$ and to $p67^{phox}$ to both cytoplasmic-membrane bound and specific granule NADPH oxidase assembly (Ambruso et al., 2004). It is plausible that pre-training values for concentration of ROS produced were a sum of both membrane and specific granule NADPH oxidase activity. Therefore, if degranulation did occur post-exercise, as previously proposed based on surface antigen upregulation, the amount of H_2O_2 produced post-exercise may not involve a contribution of specific membrane NADPH oxidase, thus accounting for the decreased post-exercise values observed (Figure 5-5).

Although the correlation matrix (Supplement I – Table D) strengthens this study's power by demonstrating significant correlations between iron parameters and immune phenotypes and function, its exploratory nature requires further analysis. When the very conservative Bonferroni correction was applied, only the correlation between CD11b and CD18 expression was significant. Therefore, it is suggested that future investigations should invest in increasing the number of sampling points to allow for a more powerful conclusion on the correlation between the variables mentioned.

The marked increase in serum hepcidin concentration observed post-exercise in this study reinforces previous findings (Newlin et al., 2012; Peeling et al., 2009a). Interestingly, the reported peak in hepcidin concentration three hours' post-exercise and steady decline reaching baseline levels at 24 hours, is a widely recognised response to increased IL-6 concentration post-exercise (Newlin et al., 2012). The samples in this study were obtained at the end of the training day (see *Methods*), when time elapsed from the first training bout was approximately seven hours. Possibly, the substantial increase in serum hepcidin seen in this study (>76%) is a cumulative effect of multiple daily training sessions. Cumulative effects of multiple training sessions in (urinary) hepcidin concentration have been previously investigated in male triathletes and endurance runners with healthy iron status (Peeling et al., 2009a). In this randomized cross over study (seven days between trials), athletes ran either one session (T1) (10x1km interval running at 90% of individual peak $\dot{V}O_2$ running velocity) or two-sessions (T2) ((1) 10km run at 70% of peak $\dot{V}O_2$ running velocity and (2) after a 12-hour overnight rest period, same protocol described in T1)). While results from this study demonstrated that a 12-hour rest period was sufficient to restore urinary hepcidin levels back to baseline, after it had been significantly elevated three hours post-exercise, with no significant discrimination between trial intensities, it does not clarify the potential cumulative effects of multiple exercise sessions performed on the same day. Taken that hepcidin responds to an increased concentration of serum IL-6, whose concentration has been reported to peak at 1.5 hours post-exercise (Bruunsgaard et al., 1997; Margeli et al., 2005; Pedersen, Steensberg, & Schjerling, 2001) it could be postulated that the adopted training schedule in this study was sufficient to induce and maintain increased levels of IL-6. Ronsen et al. (2002) have shown that a second session of exercise (of same duration and intensity) after three hours of rest of an initial session (75 minutes at 75% $\dot{V}O_{2max}$), evokes a more pronounced peak in plasma IL-6 response than a single training session. This group further demonstrated that while prolonging the rest time between trials to 6-hours attenuated the IL-6 peak plasma concentration

observed with a shorter rest period, a second training session still produced a greater peak in IL-6 than a single daily session, highlighting significant cumulative effects of training in cytokine response. Further, Roecker et al. (2005) have classified athletes as 'responders' and 'non-responders' based on changes in hepcidin concentration. Individual analysis showed that four out of the seven athletes tested were responders (hepcidin response was considered high when an increase greater than 20% from pre-exercise values was observed), similar percentage found by Newlin et al. (2012).

Finally, attention must be paid to further changes in iron parameters. Decreases in serum iron may lead to tissue ID and hinder erythrocyte production and consequently O_2 delivery to the exercising musculature. Transferrin saturation values observed in this study might not have shown a statistically significant decline, but the 20% decrease from pre- to post-training requires further monitoring, as transferrin saturation less than 16% has been correlated with deficient erythropoiesis (Chatard, Mujika, Guy, & Lacour, 1999). Two out of the seven athletes presented values equal to or lower than this level at the end of the training day. The literature suggests that such decrease may persist as Fallon, Fallon, and Boston (2001) demonstrated that female soccer players had a significant decrease in transferrin saturation at the cessation of a heavy training week ($21\pm7\%$) compared to baseline values ($36\pm13\%$). As iron status was not investigated on a daily basis it remains unclear if the post-training values returned to baseline; reiterating the need for monitoring iron status in elite female athletes.

The importance of periods of increased training load for performance enhancement is undeniable. However, it is becoming evident that a balance between the imposed stress and recovery are of uttermost importance to avoid time lost to illness hence ensuring the so-desired performance gains. This study has demonstrated that a two-week intensified training camp was sufficient to perturb both the immune system and iron metabolism in elite female kayak athletes. Redistribution of leucocyte sub-populations with varying phenotypes may be correlated with alterations in immunological function throughout a HI training period. Further, it has become clear that for some parameters, such as the expression of surface antigens related to neutrophilic functions, a 24 hour 'rest' day is insufficient to return measured values to pre-training camp values. The daily variation in immune cell distribution may come to burden other systems such as cellular turnover and, consequently, increase bone-marrow activity (Zhao et al., 2012). As it is still unknown if the constant demand on cellular turnover may be sustained as functional-related antigen

expression in neutrophils showed declines throughout the camp compared to baseline values, further research is required.

Supplement I – Chapter 5

Table A – Training schedule Gold Coast Camp April 2015

	Date	M	T	W	Th	F	S	Su	M	T	W	Th	F
		20	21	22	23	24	25	26	27	28	29	30	1
				TDay 1		TDay 2			TDay 3		TDay 4		
Training Session	AM1			On water testing 4x750m (SR:65,75,80 ,85) + 1x500m max 12 min base	2x8min, 6x6min@T2 12 sec of each 2 min @T5	Force testing (K4) SR: 90, 100, 110, 115-120			20-30min full race warm-up 5 (20sec, 40sec off, 40sec on 20sec off, 20sec on) -- on's at race pace off's at T2/T3 7min base.	Force Testing (5x300m)	6x400m. Starts and transitions + 20min individual float	4x750m @ 80 SR 6 min base + 6x250m on 3 min base T5	Time trials 3x500m on 30min base
	AM2				Drills and skills session - 2x750m broken (250m on/off/on) + change of pace 3x45sec 4 starts	K2 Warm up to 300m course 7x300m @110SR on 6min base	7min base T4 efforts 4 & 8 max (standing starts)		K4 drills and skills session -- 2x750m broken (250m on/off/on) + change of pace 2 (3x45sec %,SR)	Warm up to 300m course 7x300m @110SR on 6min base		Drills and skills session - 4x750m broken (250m on/off/on) + 4 starts	
	PM			Gym + resistance 1 ball 6x15sec on 1.45 off		Gym + 30min cross training			3 pm gym + resistance 1 ball 3x20sec on 1.40 off. Strap only 3x30sec on 1.30 off (power paddling)	4x6min @75-80 SR on a 12min base	Gym + resistance 1 ball 6x15sec on 1.45 off		Gym + 30min cross training

SR= Stroke rate. T = refers to training zones (table 5-1 in-text)

Table B – 750m On-water step test

Athlete	Stroke Rate	Time (min:s.ms)	Velocity (m/s)	eWPS (%)	Pace (min:s.ms)	Heart Rate (bpm)	Lactate (mM)
A001	67	03:45.10	3.33	33.1	02:30.15	170	2.1
	78	03:29.38	3.58	35.3	02:19.66	179	5.3
	81	03:26.97	3.62	35.1	02:18.12	180	6.8
	87	03:23.41	3.62	34.7	02:15.50	181	10.9
	107	02:08.48	3.89	33.0	02:08.53	181	13
A002	56	04:07.22	3.03	29.8	02:45.02	147	1.4
	69	03:57.09	3.16	27.4	02:38.23	150	2.6
	75	03:52.23	3.23	27.0	02:34.80	157	3.0
	83	03:42.28	3.37	27.7	02:28.37	167	5.2
	102	02:18.29	3.62	27.9	02:18.12	174	11.0
A003	65	03:52.45	3.23	31.1	02:34.80	150	1.3
	71	03:39.16	3.42	33.8	02:26.20	160	1.8
	78	03:32.41	3.53	33.8	02:21.64	168	2.8
	85	03:25.63	3.65	34.3	02:16.99	177	3.8
	101	02:09.83	3.85	33.9	02:09.87	180	7.5
A004	68	03:49.77	3.26	30.6	02:33.37	156	1.8
	74	03:38.48	3.43	32.7	02:25.77	168	3.6
	79	03:31.47	3.55	34.0	02:20.85	174	4.9
	85	03:24.85	3.66	34.6	02:16.61	180	6.6
	108	02:07.30	3.93	33.7	02:07.23	191	15.1
A005	66	03:46.79	3.31	33.0	02:31.06	N/A	2.4
	75	03:32.49	3.53	35.2	02:21.64	N/A	3.3
	80	03:30.27	3.57	34.1	02:20.06	N/A	4.0
	83	03:25.92	3.64	34.9	02:17.36	N/A	5.7
	104	02:10.00	3.85	32.9	02:09.87	N/A	8.3
A006	65	03:52.78	3.22	30.8	02:35.28	135	1.2
	75	03:38.06	3.44	32.6	02:25.35	138	2.5
	84	03:24.78	3.66	35.0	02:16.61	158	4.5
	85	03:26.79	3.63	33.8	02:17.74	168	4.9
	102	02:07.47	3.92	35.4	02:07.55	176	6.8
A007	69	03:50.26	3.26	30.1	02:33.37	150	2.2
	77	03:33.39	3.51	33.7	02:22.45	158	3.0
	83	03:26.79	3.63	34.6	02:17.74	170	4.0
	88	03:21.54	3.72	35.1	02:14.41	176	5.4
	106	02:07.08	3.93	34.4	02:07.23	178	8.0

Data collected by AIS senior physiologist

Table C – Acute changes in neutrophil phenotypical expression and distribution

			Mean	Std. Deviation	Mean % Change	t	Sig. (2-tailed)	Effect size (ES)
CD11b MedianFI (AU)	TDay1	Pre	4940	916	14.2	-1.047	.335	-.118
		Post	5593	1856				
	TDay2	Pre	3489	1063	12.7	-1.400	.211	-.083
		Post	3921	1540				
	TDay3	Pre	3582	920	-13.5	1.597	.161	.183
		Post	2969	756				
	TDay4	Pre	3889	1108	9.5	-.096	.927	-.013
		Post	3943	933				
CD16 MedianFI (AU)	TDay1	Pre	8511	1913	-6.7	0.673	.526	.049
		Post	8028	3047				
	TDay2	Pre	7363	1871	5.9	-1.848	.114	-.050
		Post	7716	1632				
	TDay3	Pre	7875	2007	-13.7	5.225	.002‡	.152
		Post	6763	1653				
	TDay4	Pre	7670	2205	0.4	.097	.926	.004
		Post	7639	2057				
CD18 MedianFI (AU)	TDay1	Pre	2486	456	11.6	-1.168	.287	-.134
		Post	2703	351				
	TDay2	Pre	1827	490	3.6	-0.406	.699	-.019
		Post	1865	506				
	TDay3	Pre	1831	359	3.4	-0.126	.904	-.014
		Post	1851	335				
	TDay4	Pre	2203	441	2.4	.270	.796	.046
		Post	2121	455				
CD66b MedianFI (AU)	TDay1	Pre	1421	256	21.7	-3.533	.012‡	-.241
		Post	1727	379				
	TDay2	Pre	1226	257	8.6	-1.590	.163	-.088
		Post	1318	263				
	TDay3	Pre	1355	238	-11.3	2.928	.026*	.201
		Post	1189	176				
	TDay4	Pre	1370	277	8.6	-.957	.376	-.083
		Post	1478	379				
CD71 MedianFI (AU)	TDay1	Pre	408	132	-1.6	.282	.787	0.120
		Post	395	76				
	TDay2	Pre	424	133	-6.9	1.115	.308	.417
		Post	378	85				
	TDay3	Pre	329	28	-3.9	.876	.415	.311
		Post	316	56		-1.014	.310	
	TDay4	Pre	364	118	22.5	-2.129	.077	.581
		Post	435	125		-2.197	.028*	
CD11b (% of Granulocytes)	TDay1	Pre	98.3	1.1	-1.1	1.200	.275	.130
		Post	97.2	3.0				
	TDay2	Pre	98.5	0.4	1.0	-6.554	.001‡	-.689
		Post	99.5	0.4				
	TDay3	Pre	99.5	0.1	-5.7	3.889	.008‡	.714
		Post	93.8	3.9				
	TDay4	Pre	98.9	0.5	0.5	-2.653	.038*	-.335
		Post	99.4	0.2				

Table C continued			Mean	Std. Deviation	Mean % Change	t	*p*	ES
CD16 (% of Granulocytes)	TDay1	Pre	93.9	3.0	2.2	-1.078	.323	-.147
		Post	95.9	3.6				
	TDay2	Pre	95.2	1.5	2.5	-8.074	<.001‡	-.396
		Post	97.6	1.5				
	TDay3 3	Pre	97.1	1.8	-6.0	3.533	.012‡	.442
		Post	91.3	4.8				
	TDay4 4	Pre	95.8	2.4	2.3	-3.227	.018*	-.334
		Post	97.9	0.9				
CD18 (% of Granulocytes)	TDay1	Pre	98.8	1.5	-0.8	0.584	.581	.091
		Post	98.0	3.2				
	TDay2	Pre	99.8	0.2	0.0	0.000	.999	.000
		Post	99.8	0.1				
	TDay3 3	Pre	99.7	0.3	-5.9	3.689	.010‡	.646
		Post	93.8	4.3				
	TDay4 4	Pre	99.6	0.3	0.3	-3.561	.012*	-.357
		Post	99.9	0.1				
CD66b (% of Granulocytes)	TDay1	Pre	98.1	1.7	-0.74	0.513	.626	.075
		Post	97.3	3.2				
	TDay2	Pre	97.9	1.4	0.94	-1.685	.143	-.220
		Post	98.8	0.7				
	TDay3 3	Pre	99.0	0.6	-6.54	3.557	.012‡	.592
		Post	92.5	4.9				
	TDay4 4	Pre	98.6	0.8	0.70	-3.250	.017*	-.286
		Post	99.2	0.4				
CD71 (% of Granulocytes)	TDay1	Pre	6.3	2.9	279.2	-2.583	.042*	-1.641
		Post	18.8	12.4				
	TDay2	Pre	7.2	4.3	-70.35	4.279	.005‡	1.568
		Post	2.1	2.1				
	TDay3 3	Pre	2.9	2.2	105.8	-0.100	.923	-.045
		Post	3.0	2.1				
	TDay4 4	Pre	5.1	3.3	-16.6	1.806	.121	.925
		Post	2.8	1.7				
Granulocytes (% of Leucocytes)	TDay1	Pre	40.9	7.7	-7.39	0.336	.748	.053
		Post	37.9	20.8				
	TDay2	Pre	33.1	5.4	32.28	-3.016	.024*	-.376
		Post	43.8	8.8				
	TDay3	Pre	37.4	8.6	32.24	-2.143	.076	-.231
		Post	49.4	17.4				
	TDay4	Pre	32.6	10.7	29.76	-1.928	.102	-.211
		Post	42.3	12.3				

* Significant at $p<0.05$ $df = 6$ for all paired T-tests performed.
‡ Significant at $p<0.01$ (Bonferroni correction)

Table D – Correlation between iron and immune markers

		Serum Iron	Tf	UIBC	TIBC	TSAT	PHAG	O.B.	CD18	CD16	CD66b	CD11b	CD71
Serum Iron	Pearson Correlation		**.834***	.639	**.804***	.730	-.357	.001	-.585	.687	-.152	-.588	-.137
	Sig. (2-tailed)		**.020**	.122	**.029**	.062	.432	.999	.167	.088	.745	.165	.769
Tf	Pearson Correlation	-.420		**.956****	**.998****	.239	-.150	.079	-.153	**.876****	.231	-.235	-.347
	Sig. (2-tailed)	.348		**.001**	**.000**	.605	.748	.867	.743	**.010**	.618	.612	.445
UIBC	Pearson Correlation	-.468	**.953****		**.971****	-.048	-.022	.135	.112	**.846***	.415	-.007	-.400
	Sig. (2-tailed)	.290	**.001**		**.000**	.919	.962	.774	.812	**.016**	.354	.988	.374
TIBC	Pearson Correlation	-.074	.885**	**.916****		.189	-.127	.104	-.095	**.867***	.274	-.187	-.352
	Sig. (2-tailed)	.875	.008	**.004**		.685	.785	.824	.840	**.012**	.551	.688	.439
TSAT	Pearson Correlation	**.894****	-.741	**-.807***	-.504		-.408	-.121	**-.845***	.102	-.530	-.750	.289
	Sig. (2-tailed)	**.007**	.056	**.028**	.248		.363	.796	**.017**	.827	.221	.052	.530
PHAG	Pearson Correlation	-.730	.642	.637	.387	**-.783***		.595	.692	.122	**.844***	**.844***	.124
	Sig. (2-tailed)	.062	.120	.124	.391	**.037**		.159	.085	.795	**.017**	**.017**	.792
O.B.	Pearson Correlation	-.266	-.436	-.304	-.464	.002	-.156		.591	.232	.721	.633	-.302
	Sig. (2-tailed)	.564	.328	.507	.295	.996	.739		.163	.616	.067	.127	.511
CD18	Pearson Correlation	.800*	-.287	-.346	-.027	.691	-.383	-.267		-.009	**.840***	**.954****	-.226
	Sig. (2-tailed)	.031	.532	.447	.954	.086	.397	.562		.985	**.018**	**.001**	.626
CD16	Pearson Correlation	.075	-.205	-.469	-.495	.290	-.019	-.311	.203		.378	.012	-.571
	Sig. (2-tailed)	.873	.659	.289	.259	.529	.968	.498	.663		.404	.979	.181
CD66b	Pearson Correlation	.488	-.230	-.423	-.256	.513	-.173	-.528	.733	**.769***		**.857***	-.156
	Sig. (2-tailed)	.267	.620	.344	.579	.239	.711	.223	.061	**.043**		**.014**	.738
CD11b	Pearson Correlation	**.861***	-.230	-.315	.036	.719	-.443	-.353	**.984****	.224	.734		-.168
	Sig. (2-tailed)	**.013**	.620	.491	.940	.069	.319	.437	**.000**	.629	.061		.719
CD71	Pearson Correlation	-.227	.702	**.826***	**.830***	-.535	.407	-.023	-.316	**-.774***	-.696	-.281	
	Sig. (2-tailed)	.625	.079	**.022**	**.021**	.216	.365	.960	.491	**.041**	.083	.541	

Blue background = pre-training samples. White background= post-training samples.
PHAG = phagocytosis. O.B = Oxidative burst.
* $p < 0.05$ ** $p < 0.01$

Chapter 6

Effects of exercise and training on immunological and iron related parameters in elite female kayak athletes during a period of "live-high, train-low" training

Introduction

'Live-high train-low' (LHTL) is a commonly adopted training methodology where athletes are exposed to a hypoxic stimulus, usually for 10-12 hours (overnight) and undertake their training in normoxic normobaric conditions (sea level). The rise to moderate altitude (2000-3000m natural or simulated i.e. hypoxic chambers) elicits physiological adaptive responses that reportedly increase performance (Hahn & Gore, 2001; Levine & Stray-Gundersen, 1997; Rusko, Penttinen, Koistinen, Vahasoyrinki, & Leppäluoto, 1995). While still under debate, such adaptations include an increase in O_2 carrying capacity (via increased erythropoiesis and increase in haemoglobin mass (Hb_{mass})) (Brugniaux et al., 2006; Wehrlin, Zuest, Hallén, & Marti, 2006), increase in $\dot{V}O_{2max}$ (Stray-Gundersen & Levine, 2008; Wilhite, Mickleborough, Laymon, & Chapman, 2013), improvement in exercise economy (Saunders et al., 2004), increase in muscle buffering capacity (Gore et al., 2001), and increase in lactate threshold (Gore, Clark, & Saunders, 2007), which are of utmost importance to sprint kayak performance (Bishop, 2000; Michael, Rooney, & Smith, 2008).

The majority of the altitude training research focuses on direct performance outcomes, such as $\dot{V}O_{2max}$ and lactate threshold, as mentioned above. Recently, focus has shifted towards haematological adaptations to hypoxia, mainly focused on erythrocyte production via EPO release, Hb_{mass} and iron related parameters (Govus, Garvican-Lewis, Abbiss, Peeling, & Gore, 2015). The increases in Hb_{mass} and sTfR concentration observed in national level triathletes after 17 days LHTL (~14 hours daily) exposure to normobaric hypoxia equivalent to 3000m altitude (Humberstone-Gough et al., 2013), highlight the effectiveness of this commonly adopted exposure methodology in eliciting an erythropoietic response. Rather than using altitude in the lead-up to competition, altitude may be used before or during heavy aerobic periods. When used prior to or during heavy aerobic training periods, the focus is on maximising Hb_{mass} which will allow the athlete to train at a higher aerobic intensity (Saunders, Pyne, & Gore, 2009). This increase in training capacity may produce the smallest worthwhile change in performance of 0.5%, which already differentiates medal from non-medal athletes (Bonetti, 2008; Borges, 2013).

Iron availability is an important determinant of physiological adaptations to hypoxia. While there is evidence of the relationship between iron metabolism and hypoxia, the

exact molecular mechanisms are still unclear (Frise et al., 2016; Govus, 2015; Peyssonnaux et al., 2008). Studies suggest that hypoxia may coordinate iron availability by regulating expression of the iron regulatory peptide hormone hepcidin through a direct pathway involving HIF factors (Peyssonnaux et al., 2008) or an EPO-dependent pathway (Kautz et al., 2014; Kautz & Nemeth, 2014; Rishi, Wallace, & Subramaniam, 2015). Hypoxia-induced EPO release stimulates the production of the hormone erythroferrone (ERFE) by erythroid precursors in the spleen and bone marrow through signal transducer and activator of transcription 5 (STAT5)(Kautz et al., 2014). ERFE acts on the liver to suppress the expression of hepcidin (Kautz & Nemeth, 2014). This enables iron storage release and increased duodenal iron absorption, thus providing sufficient iron for the (reportedly three-fold - Faura et al., (1969)) increase in erythropoietic demand.

However, when an athlete adds the extra stressor of hypoxia on top of routine training there is a dichotomy in maintaining both functionally-available iron and iron stores. The hypoxic stimulus down-regulates hepcidin expression (Jeong et al., 2005; Yan et al., 1995) while exercise-induced increase in inflammation markers, such as IL-6 and IL-1, up-regulates hepcidin expression (Lee et al., 2005; Nemeth et al., 2004; Sim et al., 2013; Wrighting & Andrews, 2006). Interestingly, hypoxia has also been reported as an inducer of oxidative stress and cytokine release (IL-6, TNF-α), which were described to occur proportionally to the severity of the hypoxic stimulus (He et al., 2014). *In vitro* studies have shown increased IL-6 transcription in cultured human endothelial cells (Yan et al., 1995) and cultured myocytes (Yamauchi-Takihara et al., 1995) exposed to hypoxic challenge (PO_2 ~ 14 Torr and 95%N_2 5%CO_2, respectively). Klausen et al. (1997) demonstrated incremental increase of serum IL-6 concentration in ten male subjects at 4350m from the first day of exposure, reaching a significant increase of 86% on day 4 compared to sea-level values. Thus, whilst hypoxia-induced low tissue O_2 triggers hepcidin down-regulation to allow iron store release to meet erythropoietic demands, increases in IL-6 and IL-1 concentration (induced by either hypoxia or exercise) have the opposite effect, up-regulating hepcidin expression (via STAT3 activation) (Lee et al., 2005).

Some studies suggest that acute systemic hypoxic exposure may alter immunological function, mostly attributed to altitude-related increases in cortisol, adrenaline and noradrenaline (Mazzeo, 2005). Comparable to changes in immunological parameters after a single bout of exercise, altitude results in significant lymphopenia and neutrophilia

in peripheral blood, as well as impairments of T-cell activation and proliferation and increases in NK lymphocyte concentration and activity (Facco et al., 2005; Karagiannidis et al., 2006). One day of hypobaric hypoxic exposure (4500 m) elicited a significant ~20% increase in circulating neutrophils (Hitomi et al., 2003). Neutrophilic CD18 expression was increased by more than 20% and the rate of spontaneous O_2^- production per 10^6 neutrophils in 15 minutes was enhanced six-fold in 12 individuals after 60-90 minutes after arrival at 3196m (helicopter ascent) (Choukèr et al., 2005). These neutrophilic responses, as well as enhanced degranulation (quantified through elastase release), were also observed in a study employing acute normobaric hypoxia (68% of SaO_2 measured through pulse oximeters) in healthy volunteers (Tamura et al., 2002).

The addition of exercise to the hypoxic stimulus may affect the immune system more significantly, stemming from a more pronounced sympathoadrenal response (Mazzeo, 2005). Wang and Chiu (2009) showed a more marked neutrophilia following moderate exercise in hypoxic (12% O_2) environment than when the same exercise protocol performed in normoxia. Further, the same group demonstrated an enhanced phagocytic and oxidative burst capacity as well as increased expression of adhesion molecules (L-selectin, LFA-1 C5aR and CD11b/CD18) in neutrophils after 50% $\dot{V}O_{2max}$ exercise protocol was performed at 12% O_2 compared to the same protocol performed at 21% O_2 (Wang & Chiu, 2009).

These studies have highlighted the effects of exercising under hypoxic conditions. However, this escapes the reality of the commonly adopted LHTL protocol, where training occurs in normoxia and the hypoxic stimulus is undertaken overnight. The few studies to date which analysed the impact of LHTL on immunological status explored mostly mucosal immunity (salivary IgA - Tiollier et al., 2005 and lymphocyte T cell redistribution; Zhang et al., 2007) . Using this hypoxic model (LHTL), we set out to observe the possible immunological changes, particularly leucocyte phenotype distribution and neutrophilic function during this specific training period. Additionally, owing to the relation between functionally available iron as well as iron stores and hypoxia and the knowledge that serum iron may be decreased post-exercise, particularly in female athletes, this study also aimed to profile the iron status of elite female kayak athletes undergoing a LHTL training camp. In the previous study performed on these athletes (see Chapter 5), it was shown that hepcidin was significantly increased from pre-training in the samples obtained post-training. Hepcidin kinetics post-exercise have been

well characterized in literature (Díaz et al., 2015; Peeling et al., 2009a, 2009b; Peeling et al., 2014); however, its possible cumulative effects in LHTL training setting has only been described recently in Govus et al. (2016) , warranting further investigation. Therefore, due to the small sample size obtained and the number of assays performed making use of these samples, this study set out to compare serum hepcidin concentration in samples obtained at the end of TDay2 and TDay3.

Methods

All methodological procedures are thoroughly detailed in the *Methodology* section of this thesis (Chapter 3). Here, methods utilised for this camp will be described briefly.

Six elite female kayak athletes (age = 25.5±3.7 years; mass = 74.5±5.3 kg; $\sum 7_{skinfold}$ = 75.2±16.7mm; $\dot{V}O_{2peak}$ = 52.1±3.3mL/kg/min) in selection to represent Australia in the 2016 Olympics participated in this study. The athletes trained at sea level and spent the night (10-12 hours) in altitude tents (Colorado Altitude Training™, Boulder, Colorado), starting at 2000m ($F_IO_2$16.5% O_2) on the first day, progressing to 2600m (F_IO_2 15.9% O_2) on the second day and reaching the equivalent to 3000m (F_IO_2 14.8% O_2) on the third day. This initial altitude acclimatization was performed in the athlete's home-base (i.e. interstate athletes) one week prior to arrival at the Gold Coast training camp (Table 6-1). Such normobaric hypoxic conditions weres achieved by addition of nitrogen, hence decreasing the percentage of available O_2 to levels as low as 14.8%. Athletes remained in the LHTL program at the simulated altitude of 3000m for a total of 18 days. The LHTL program was structured and overseen by AIS staff, with no input from researchers. Also following AIS standards to maintain athletic nutrient (trace element) intake, athletes were supplemented once daily with commercially available iron supplements containing 325mg dried ferrous sulphate BP (equivalent to 105 mg elemental iron) and 500mg ascorbic acid (Ferro Gard C, Abbott Laboratories, Botany Bay, Australia). As one of the athletes discontinued the altitude component she was excluded from the study. The remainder of the athletes in this cohort had undertaken the LTHL protocol on at least two occasions prior to this exposure.

All training protocols performed during the camp were planned and executed by the AIS coaches with no direct supervision or intervention from the investigators. Training load

was quantified through AIS-adopted methodology of sessional RPE (Borg scale 6-20) multiplied by the duration of the training. As part of their habitual training program during the 10-day camp the local athletes (n=3) performed laboratory testing of haemoglobin mass using the carbon monoxide rebreathing technique (reviewed in Gore et al., 2013). This test was performed by an AIS staff member who had been performing this test for at least four years and had a typical error of measurement of 1.9% for this test.

Blood Collection

Prior to any blood collection the athletes had been acclimatizing to altitude exposure for one week in their respective training headquarters (Table 6-1). Capillary blood (300µL) was obtained from the earlobe, after 5-10 minutes of application of thermogenic cream (Finalgon®) prior to ('pre-training' 05:00-06:30 hours) and at the end of the training day ('post-training' 15:00-16:30 hours) in each of the testing days of the training camp (assigned on Table 6-1). Since the athletes did not train under the same protocol on the last two days of the camp and as athletes not based in Queensland returned to their home-state, testing dates were selected to ensure that all athletes tested were under the same training protocol (Table 6-1).

Table 6-1 – Training and testing schedule for camp held in June 2015.

Date	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
(June 2015)	T	W	Th	F	S	Su	M	T	W	Th	F	S	Su	M	T	W	Th	F	S
Altitude Tent																			
Training Camp																			
Athlete Arrival/ Departure																			
Hb_{mass} *																			
Capillary samples (Immune)									TDay1		TDay2			TDay3		TDay4			
Capillary samples (Iron)																			

* Hb_{mass} was only assessed on local athletes (n=3).
TDay – Testing days. The numbers assigned on the capillary sample testing days are later referred to in text.

Phenotypical assessment

Briefly, whole blood obtained from a capillary site (20µL) was stained for 15 minutes in the dark with monoclonal antibodies (all from BD Biosciences, California, USA) in two separate tubes: tube 1 contained CD3 (FITC), CD4 (PE-Cy7), CD8 (PerCP-Cy5.5), CD19

(APC-H7), CD56 (PE) and CD71 (APC) and the second tube contained CD11-b (APC-Cy7), CD66b (PerCP-Cy5.5), CD16b (PE), CD18 (FITC) and CD71 (APC). Erythrocytes were lysed with NH_3Cl solution for 10min, centrifuged at 300 x g for 5 minutes and washed with PBS. Cells were fixed with 1% formaldehyde and analysed through a FACSVerse flow cytometer (BD Biosciences, California, USA) immediately.

Neutrophilic function

Phagocytosis of FITC labelled *E.coli* and PMA-stimulated oxidative burst capacity were performed independently using capillary whole blood (25µL) in previously described in the *Methodology* section (Chapter 3).

Iron Status

Serum obtained from the capillary samples was diluted 1:3 in NaCl (0.9%) solution (final volume 90µL) and SI, TfR and UIBC were analysed through an automated biochemical analyser (Cobas Integra® 400 Plus Roche). TIBC was calculated by adding UIBC to SI. TSAT was calculated by the formula (SI÷TIBC) x 100.

IL-6

Serum IL-6 concentration was determined by commercially available Human Quantikine® IL-6 ELISA kit (R&D Systems) on samples obtained pre- and post-training following the manufacturer's protocol.

Hepcidin

Serum hepcidin concentration was quantified through the use of a commercially available Human Hepcidin Immunoassay Quantikine® ELISA kit (R&D Systems) following manufacturers' protocol.

Statistical Analysis

The effect of the exercise per day was analysed through paired samples T-test comparing pre-training samples to end of the day samples. If data violated assumptions non-parametric Wilcoxon Signed Rank test was applied. After Bonferroni correction for

multiple comparisons, significance was attained where $\alpha<0.05/4$. Effect size (ES) was calculated in every paired sample t-test according to Cohen (1988), as previously described. ES was considered 'small'<0.2, 'medium'=0.5 and 'large'>0.8. A Pearson's product-moment correlation coefficient was used on samples obtained prior to and at the end of TDay2 to assess the possible relationship between iron and immune parameters. To highlight the effects of the overnight hypoxic exposure in each of the parameters analysed, the samples obtained prior to training in each testing day (fixed effect) were modelled through mixed model analysis with AR1 structure for repeated measures (day) accounting for each individual athlete through random intercept and slope (day). Finally, through a linear mixed model phagocytic and oxidative burst capacity were assessed using training day as a fixed effect and the phenotypical distribution as a covariant, as detailed previously, with significance set at $\alpha<0.05$.

Results

Overnight exposure to hypoxic environment throughout the entirety of the camp had significant haematological effects on the athletes. This is corroborated by the significant 4% increase in the Hb_{mass} (p=0.037) relative to body mass prior to and at the end of the training camp (Table 6-2).

Table 6-1 – Haemoglobin mass prior to and at the end of LHTL camp

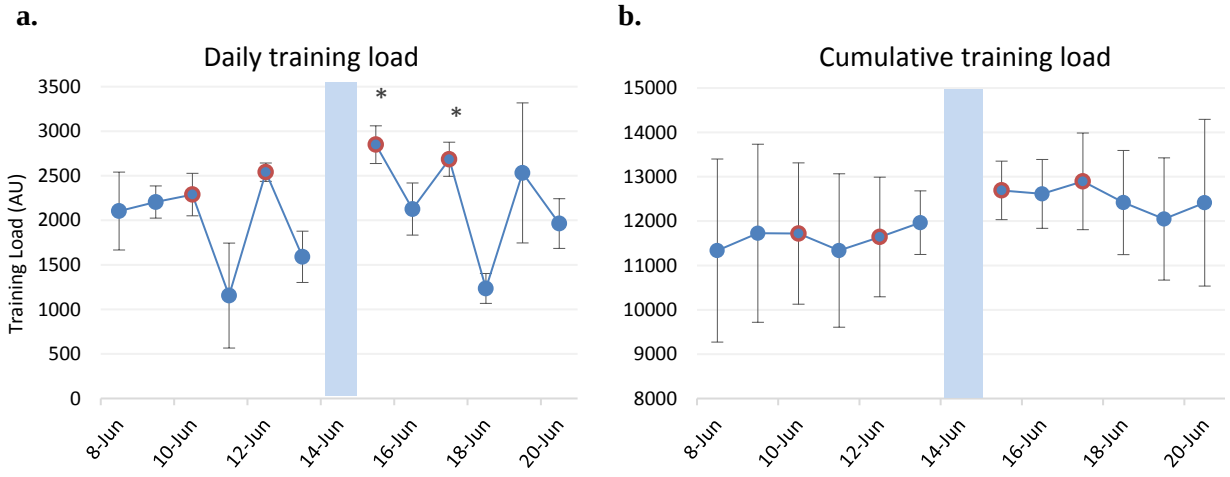
	Pre-Camp		End of Camp		% Change	*t*	*P*	ES
	Mean	SD	Mean	SD				
Hb Mass (g)	812.5 ±	82.7	842.6 ±	87.6	3.7	-4.28	.050*	0.35
Hb Mass (g/kg)	11.2 ±	0.3	11.7 ±	0.4	4.4	-5.07	.037*	1.31

Significance set at $p\leq0.05$ in a two-tailed paired sample t-test (df=2). ES = effect size (Cohen's d)

Training load

Training load increased significantly throughout the training camp (Figure 6-1). Training loads on TDay3 (2879±195 AU p<0.001) and TDay4 (2685 ±192 AU p=0.013) were significantly higher than the load imposed on TDay1 (2261± 215AU).

Figure 6-1 – Training load throughout LHTL camp



Data points represent mean values and error bars represent SD.
Testing days are assigned with red border. * Significant difference (p<0.05) from baseline (TDay 1)

Table 6-2 – Distribution of training distance and duration per training zone

	T1	T2	T3	T4	T5	T6	T7	T8
Total distance on-water (kilometres)	40	69	49.5	15.5	9.7	6.4	2.5	0.2
Total time (minutes)	228	407	227.5	69.8	38.8	24	8.8	0.7

Pre- to post-training variations

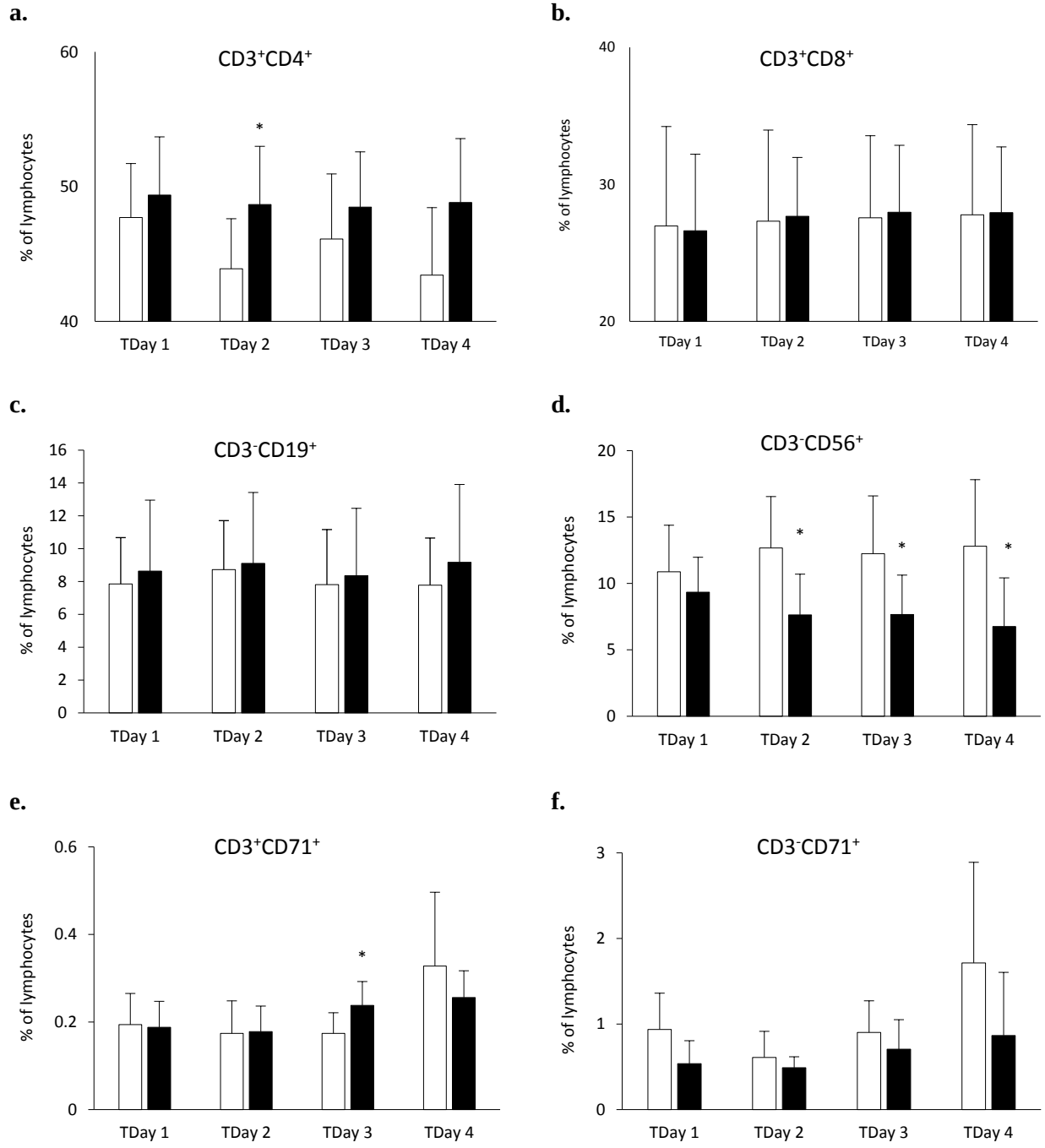
Variations in phenotype of circulating leucocyte subpopulations and neutrophilic function from pre- to post-training were assessed through paired samples t-test.

Phenotypical distribution

The percentage of $CD3^+CD4^+$ lymphocytes was significantly increased from pre (43.9±3.7%) to post-training (48.7±4.3%) on TDay2 (p=0.016, ES=1.2) (Figure 6-2a). Percentage of NK lymphocytes ($CD3^-CD56^+$) showed a significant decrease from pre- to post-training on TDay2 (12.7±3.9% to 7.6±3.1% p=0.014, ES=1.4), TDay 3 (12.2±4.3 to 7.7±3.0% p=0.002, ES=1.2) and TDay4 (12.8±5.0 to 6.8±3.7% p=0.003, ES=1.4) (Figure 6-2d). Cytotoxic T lymphocytes ($CD3^+CD8^+$) and B lymphocytes ($CD3^-CD19^+$) did not show any significant variations from pre- to post-training (Figure 6-2b and 6-2c, respectively). Further, CD71 expression in the $CD3^+$ lymphocyte sub-population was significantly increased post-exercise on TDay3 compared to pre-training samples obtained that same day (p=0.002, ES=1.2) (Figure 6-2e). $CD3^-$ lymphocyte-sub

population did not show any significant variation in percentage of cells positive for CD71 between samples obtained prior to and at the end of the training day (Figure 6-2f).

Figure 6-2 – Lymphocyte distribution pre- and post-training

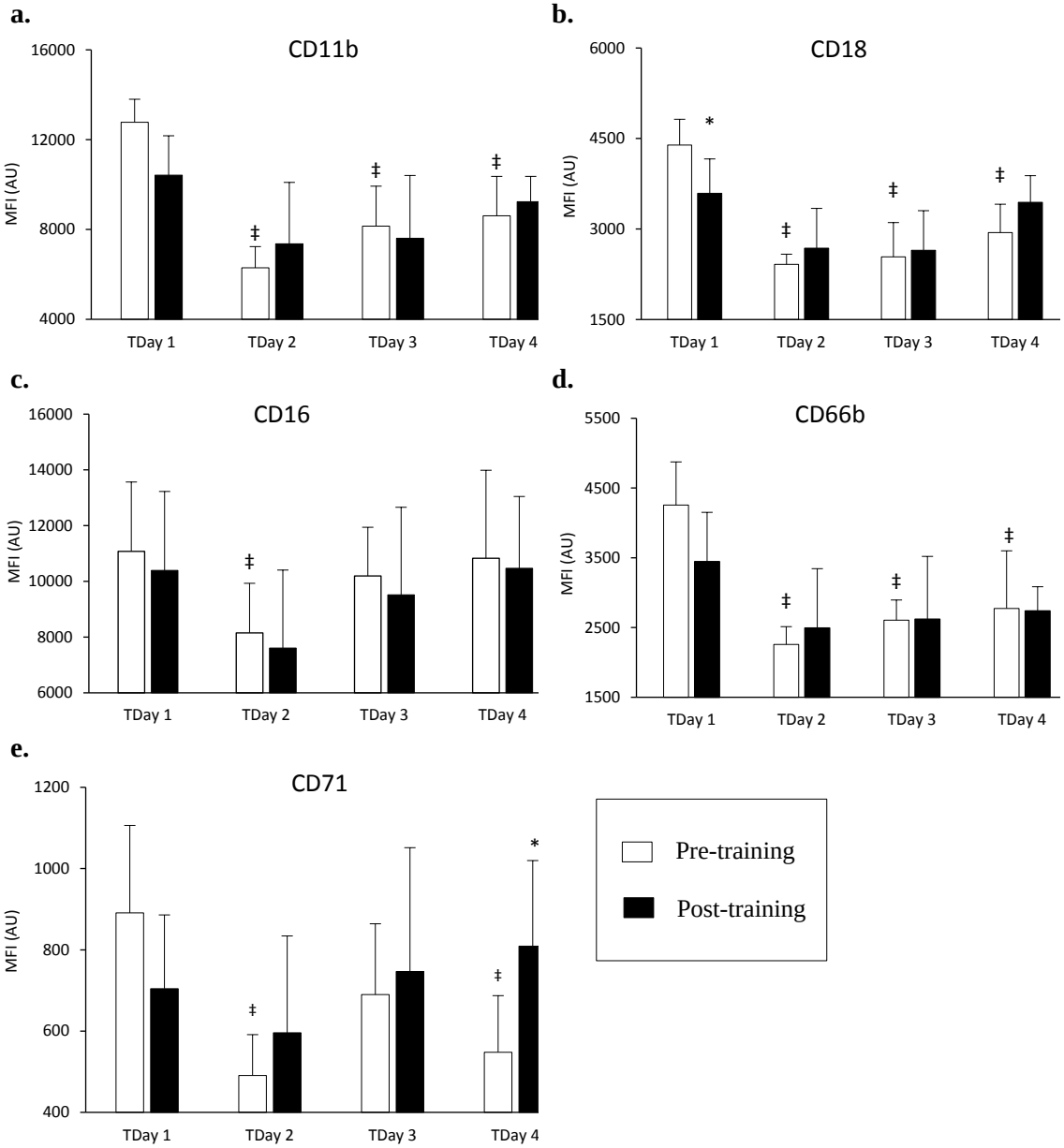


Solid bars represent mean values and error bars represent SD.
* Significant difference (p<0.01) between pre- and post-training values.

Disturbance in granulocyte phenotypical distribution was only observed on the first training day (TDay1) where the expression of CD11b (p=0.021, ES=1.7), CD16 (p=0.042, ES=0.26) and CD66b (p=0.038, ES=1.2) was down-regulated post-training (Figure 6-3a-c). Also in TDay1, there were significant decreases (p=0.005, ES=1.6) in the expression

of CD18 from pre-training (4391±428 AU) to post-training (3591±570 AU) (Figure 6-3d). Different to the post-training decrease observed on TDay 1, the expression of CD71 by neutrophils on TDay2, 3 and 4 showed a trend towards an increase post-training compared to pre-training values. On TDay4, there was a significant up-regulation of CD71 in neutrophils from pre- to post-training (548±139 AU to 809±210 p=0.012, ES=1.5) (Figure 6-3e).

Figure 6-3 – Phenotypical expression of granulocytes pre- and post-training

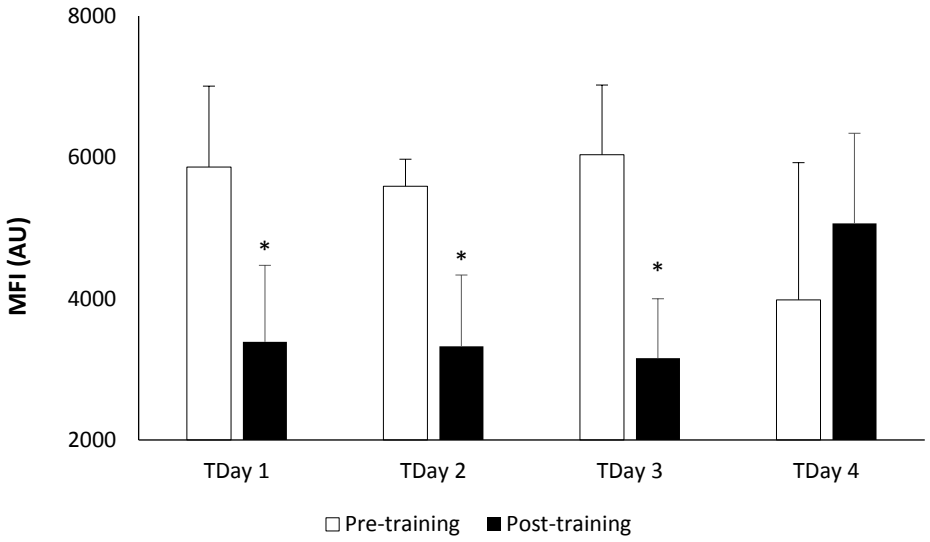


Solid bars represent mean values and error bars represent SD.
* Significant difference (p<0.01) between pre- and post-training values.
‡ Significant difference (p<0.05) between pre-training values

Granulocytic function

Phagocytic function of granulocytes, measured through FITC-labelled *E.coli* ingestion (MFI), was decreased by more than 40% from pre- to post-training day samples on the first three testing days (TDay1 p=0.017, TDay2 p=0.013, and TDay3 p<0.001) (Figure 6-4). This trend was not observed on TDay4. The percentage of neutrophils involved in *E.coli* ingestion did not differ significantly from pre- to post-training samples.

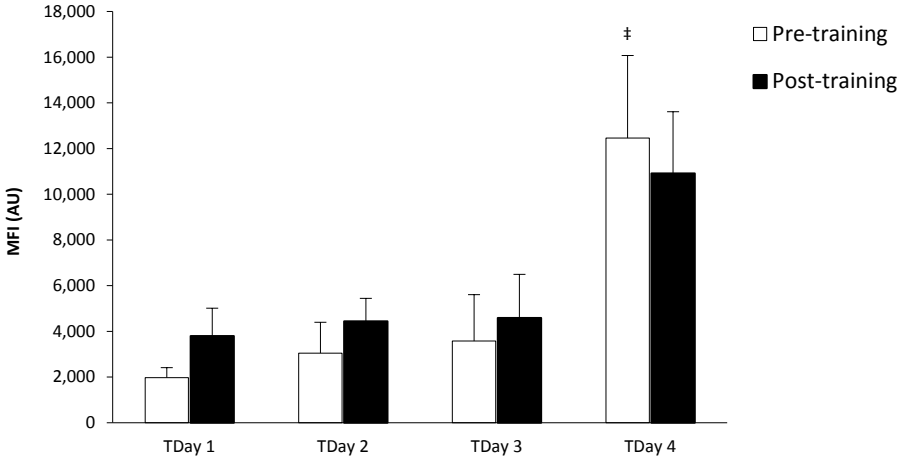
Figure 6-4 – Phagocytosis of *E.coli* by granulocytes



Solid bars represent mean values and error bars represent SD.
*Significant difference (p<0.01) between pre- and post-training values.
‡ Significant difference (p<0.05) between pre-training values compared to baseline (TDay 1)

A trend towards an increased capacity of stimulated granulocytes to produce H_2O_2 from pre- to post-training (measured by MFI) was observed on the first three testing days (TDay1 p=0.032, ES=2.2; TDay2 p=0.04, ES=1.2; TDay3 p=0.354, ES=0.5) (Figure 6-5). The last testing day was marked by an increase in both pre- and post-training sample compared to previous testing days. Contrary to the other testing days, post-training samples on TDay4 were decreased, albeit without reaching statistical significance (p=0.454, ES=0.5) (Figure 6-5). The percentage of neutrophils producing H_2O_2 was increased at the end of training TDay1 by 40% of pre-training values (p=0.031, ES=2.4). This trend was seen in every other testing day, in lesser magnitude, without reaching statistical significance (data not shown).

Figure 6-5 – Granulocyte stimulated oxidative burst capacity



Solid bars represent mean values and error bars represent SD.
*Significant difference (p<0.01) between pre- and post-training values.
‡ Significant difference (p<0.05) between pre-training values compared to baseline (TDay 1)

Iron parameters

The effects of one training day (TDay2) on serum iron parameters showed a trend towards a decrease from pre- to post-training in serum iron, TIBC and TSAT. By contrast, there was an increase in unsaturated iron binding capacity and transferrin concentration; the latter was the only measured iron value that showed significant statistical variation, increasing from pre (28.43±5.01 μmol /L) to post (30.29+4.89 μmol /L) exercise (p=0.007, ES=0.38).

Table 6-3 – Iron related parameters pre- and post-training day

	Pre		End		Mean % Change	t	*p*	ES
	Mean	SD	Mean	SD				
Serum Iron (μmol/L)	18.0	± 6.2	12.6	± 6.5	-30.0	1.14	.316	-0.85
Transferrin (μmol /L)	28.4	± 5.0	30.3	± 4.9	6.6	-5.06	.007*	0.38
UIBC (μmol/L)	31.6	± 5.2	36.1	± 10.9	14.1	-1.09	.338	0.55
TIBC (μmol/L)	49.6	± 9.5	48.7	± 8.8	-1.9	1.21	.292	-0.10
TSAT (%)	35.8	± 6.5	26.4	± 15.7	-26.1	1.01	.372	-0.84

* Significance set at p<0.05 in a two-tailed paired sample t-test (df=4). ES = Effect size (Cohen's d)

Iron status was correlated with immune phenotypes and neutrophilic functions pre- and post-training on the second day (TDay2) of the camp. Whilst low correlations were observed between iron parameters in serum, expression of CD71 on granulocytes correlated significantly with phenotypical and functional-related markers CD16 (r(5)=.941 p=0.017), CD66b (r(5)=.987, p=0.002) and CD11b (r(5)=.950, p=0.013). Interestingly, these correlations were only observed in the samples obtained at the end of training day (Supplement II - Table C).

Values for IL-6 were below the detectable range of the adopted assay (3.1-300pg/mL) in both pre- and post-exercise samples and therefore data were not shown. Hepcidin concentration was not significantly different between the two post-training day samples analysed (TDay2 6.2±1.0mM; TDay3 6.3±0.7nM p=0.829, ES= 1.3).

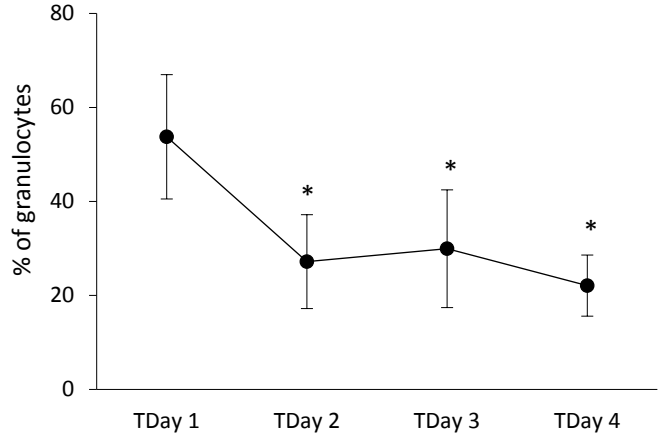
Daily variations

Phenotypical distribution

Phenotypic expression in neutrophils in the samples obtained prior to training varied between days (Supplement II- Table B). CD11b expression was decreased from baseline sample (pre-TDay1) on testing TDay2 (p=0.002), TDay3 (p<0.001) and TDay4 (p=0.007) (Figure 6-3a). Expression of CD18 followed the same trend, with significant decrements from baseline in TDay2 (p=0.001), TDay 3 (p<0.001) and TDay4 (p=0.001) (Figure 3b). CD66b was also reduced from baseline on TDay2 (p=0.006), TDay3 (p=0.003) and TDay4 (p=0.006) (Figure 3d). CD71 expression in granulocytes showed decreased values from baseline (891±215 AU) on TDay2 (491±100 AU p=0.015) and TDay4 (548±139 AU p=0.018) (Figure 6-3e).

The percentage of circulating granulocytes positive for CD11b, CD18, CD16 and CD66b did not change significantly throughout the training camp. However, there was a significant decrease in circulating CD71-positive granulocytes on samples obtained prior to testing days TDay2 (27.2±10.0% p=0.001), TDay3 (30.0±12.5% p<0.001), and TDay4 (22.1±6.5% p=0.001), compared to baseline values (53.8±13.2%) (Figure 6-6).

Figure 6-6 – Percentage of circulating granulocytes positive for CD71



Data points represent mean values and error bars represent SD.
* Significant difference (p<0.05) from baseline value (TDay1)

Granulocytic function

When comparing all samples obtained pre-training, the percentage of granulocytes participating in phagocytosis did not differ from baseline to subsequent training days. However, *E.coli* ingestion was significantly decreased (p=0.033) on the last training day (TDay 4 3983±1941AU) when compared to baseline (TDay 1 5863±1144AU) (Figure 6-4).

Pre-training granulocytic capacity for ROS production was significantly increased on the last testing day (10812 ± 612) compared to all previous testing days (Day 1 1976±436AU; Day 2 3049±1349AU; Day 3 3575±2032AU - all p <0.001) (Figure 6-5).

Discussion

This unique study assessed phenotypical distribution of leucocyte sub-populations and granulocytic function in elite female kayak athletes during a planned period of LHTL. The introduction of the hypoxic challenge to the analysed training period elicited a decrease on the resting concentration of circulating granulocytic phenotypes, particularly CD11b/CD18, CD66b and CD71 throughout the camp. Granulocytic phagocytosis, in the resting samples however, seem to have been restored from observed post-training decreases.

There was a paucity in the redistribution of the circulating T-lymphocyte subset ($CD3^+$) from pre- to post-training in this study, which may be explained by the successful acclimatization of athletes prior to the onset of the training camp (Figure 6-2). Mazzeo (2005) has described acute hypoxia to cause a significant decrease in circulating $CD3^+CD4^+$ concentration as well as decline in activation and proliferative ability of T-lymphocytes. This decline in adaptive immune response lead the author to suggest that a one week acclimatization allowance be granted for elite athletes to avoid infection (Mazzeo, 2007). Zhang et al. (2007) found that the $CD3^+CD4^+/CD3^+CD8^+$ lymphocyte ratio in soccer players exposed to 28 days of simulated normobaric LHTL (F_IO_2 14.2%) was significantly decreased on days 14 and 28 compared to baseline values. Such a decrease was not observed in this study (data not shown). As athletes underwent acclimatization prior to the camp and testing days, the acute effects of hypoxia on $CD3^+$

lymphocyte sub-populations described in the literature were not observed. The distribution of NK lymphocytes however, was affected by exercise, with values obtained post-training significantly lower than those obtained prior to training (Figure 6-2d). This has been demonstrated by others who indicate that the intensity-dependent exercise-induced muscle tissue injury is associated with decreases in post-exercise concentration of circulating NK lymphocytes, which may last up to 8 hours after cessation of the activity (Del Giacco et al., 2004; Kakanis et al., 2010; Northoff, Enkel, & Weinstock, 1995). The distribution of NK cells in the circulation post-exercise has been reported to return to pre-exercise values after 24 h of an acute bout of intense exercise. Analysis of the effects of training day on NK cells (pre-training samples only) through linear mixed model adds to previous findings as pre-exercise values did not differ significantly between testing days. Moreover, since values obtained in this study reflect other studies that analysed samples after a 24-h rest period post-exercise (Kakanis et al., 2010), it may be concluded that the overnight hypoxia does not seem to play a significant role in NK cell redistribution.

Post-exercise variation in the expression of specific neutrophilic phenotypes have been previously described (Gabriel & Kindermann, 1998; Peake, 2004). Interestingly, on the first testing day, all neutrophilic phenotypes analysed showed a decrease in expression from pre- to post-training. This however, was not observed in the subsequent testing days. The lack of statistically significant variation of neutrophilic phenotypical expression from pre- to post-training on TDay2, 3 and 4 observed in this study may point towards a less pronounced neutrophilic activation post-training than what has been reported in the literature (Gabriel & Kindermann, 1998; Peake, 2004).. Gabriel and Kindermann (1998) showed increased expression of both CD11b ($p<0.01$) and CD18 ($p<0.05$) in neutrophils from 19 cyclists and triathletes following a cycling-to-exhaustion trial at 110 % of individual anaerobic threshold. Gray et al. (1993) showed an increased expression of CD11b post-intensive exercise, closely related to degranulation of secretory vesicles and secondary granules, which are known sub-cellular locations of CD11b. The positive correlation between CD11b and CD18 observed only in the post-training samples (Supplement II- Table C) strengthens this argument and the validity of the assay employed as an index of activation, as the combination of these markers make up the α and β chain of the Mac-1 integrin protein, respectively. The correlation observed between CD11b and CD66b in the post-exercise samples may also be attributed to the previously described post-exercise degranulation, although values for these phenotypes post-training were not significantly different from pre-training values.

While training itself did not seem to perturb neutrophilic phenotypical distribution in the peripheral circulation (pre- vs post-training), overnight exposure to hypoxia caused a significant decrease in the expression of CD11b, CD18 and CD66b by circulating neutrophils (Figure 6-3). It is important to highlight that resting values on TDay4 for these markers were significantly decreased from values obtained prior to the LHTL camp (TDay1). The functional importance of CD11b in neutrophilic adherence (combined with CD18), phagocytosis and oxidative burst has been widely demonstrated and it is evident in leucocyte adhesion molecule deficiency (LAD), as LAD patients suffer recurrent infections and have impaired inflammatory exudate formation (Berton, Yan, Fumagalli, & Lowell, 1996; Coxon et al., 1996; Diacovo, Roth, Buccola, Bainton, & Springer, 1996; Kuijpers et al., 2007). Further, Coxon et al. (1996) showed complete inefficiency of CD11b knockout mice neutrophils in phagocytosing serum-opsonized paraffin oil droplets and a 60% reduction in oxidative burst (through dichlorofluorescein diacetate oxidation) compared to their wild-type counterpart. Not coincidently, TDay4 also demonstrated a decreased capacity of neutrophils to ingest *E.coli* (measured by MFI - Figure 6-4). Further studies should extend the testing schedule (i.e. days after completion of camp) to unravel the kinetics of functional surface antigen expression after a LHTL camp as persisting decrements in such antigens may impair vital neutrophilic functions and increase the risk of illnesses.

Interestingly, training had a negative impact on neutrophil phagocytic capacity, as observed by the decreased values post-training compared to pre-training samples obtained on TDay1, 2 and 3. Such a trend is often observed in periods of intensified training (Nieman et al., 2014) and at the end of prolonged endurance (>1.5 hours) exercises (Chinda et al., 2003; Chishaki et al., 2013). However, after spending the night in hypoxic chambers phagocytic capacity was restored or even enhanced compared to the baseline values obtained prior to training (TDay1)(Figure 6-4). The increase in phagocytic capacity post-hypoxic exposure may be related to neutrophilic adaptability and capacity to perform its functions in hypoxic environments, such as inflammatory sites, where oxygen tensions have been reported to be as low as 4 mmHg (Caldwell et al., 2001). Further evidence of such capacity comes from neutrophilic reliance on anaerobic glycolysis as the main ATP-producing pathway for most of its cellular functions (Cramer et al., 2003; Gale & Maxwell, 2010). HIF-1α has been shown to govern the shift towards such pathway in hypoxic environments by increasing expression of glucose transporters (Ebert, Firth, & Ratcliffe, 1995) and glycolytic enzymes (Semenza, Roth, Fang, & Wang,

1994), pyruvate dehydrogenase kinase 1 (Kim, Tchernyshyov, Semenza, & Dang, 2006; Papandreou, Cairns, Fontana, Lim, & Denko, 2006) and altering expression of cytochrome c oxidase components (Fukuda et al., 2007). The addition of the nightly hypoxic exposure may have created a conducive environment for neutrophils, with reduced tissue oxygen and increased levels of circulating inflammatory markers. Due to the constant exposure of myeloid cells to hypoxic environments, hypoxia alone is incapable of promoting HIF-1α stabilization (Gale & Maxwell, 2010). Thus, as an evolutionary/protective measure, in myeloid-derived cells, only the combined signalling of hypoxia and inflammatory markers (i.e. TNF and LPS) enhances HIF-1α accumulation promoting binding to hypoxia responsive elements, consequently increasing transcription of target genes involved in responses such as maturation, activation and survival (Cummins, Keogh, Crean, & Taylor, 2016). HIF1- α has been described to delay neutrophilic apoptosis through a 'cross-talk' with pathways such as NF-κB and PI3 kinase-Akt-mTOR, although specific molecular mechanisms are not fully elucidated (Rius et al., 2008; Walmsley et al., 2005). Knowing that hypoxia delays neutrophilic apoptosis, the return of the phagocytic capacity observed on TDay2 and TDay3 (comparable to baseline), after a significant decline immediately after training, may be hypothesised to be due to the prolonged presence of the mature neutrophils in the circulation (Figure 6-3). These are more capable of phagocytosis when compared to their naïve counterparts (band neutrophils i.e. left shift), well described to be marginalised post-exercise (Suzuki et al., 2003).

Curiously, the last training day presented a significant increase in the capacity of neutrophils to produce H_2O_2 (Figure 6-5). ROS formation in ROS-producing cells (ie neutrophils, endothelial cells) is dependent on the assembly of NADPH oxidase. Cassatella et al. (1990) reported a 3-fold increase in mRNA levels of $gp91^{phox}$ in IFN-γ-treated neutrophils. Other stimuli, such as LPS and iron, have been reported to increase gene transcription of haem-containing, catalytic NADPH oxidase-component, $p22^{phox}$ in human aortic endothelial cells (HAEC) (Li & Frei, 2009). Increases in NADPH oxidase components augments NADPH oxidase activity, consequently increasing cellular capacity for ROS generation. Further, HAEC incubation with TNF-α, which, similar to LPS, is known to up-regulate DMT1 in endothelial cells, has also been shown to increase NADPH oxidase activity two-fold (Li & Frei, 2009). It may be postulated that NADPH oxidase in neutrophils responds to the above mentioned stimuli in the same manner as endothelial cells, however, confirmation through further investigation is needed.

In this study, the last training day was marked by a decrease in CD71 expression on neutrophils (Figure 6-3e), which indicated a decreased requirement for iron. As the athletes were taking iron supplements, it may be hypothesized that the intra-cellular iron stores on this testing day were saturated. Li and Frei (2009) proposed that labile intracellular iron may induce oxidative stress which stimulates redox-sensitive transcription factors NF-κB and AP-1 (Manea, Manea, Gafencu, & Raicu, 2007; Manea, Manea, Gafencu, Raicu, & Simionescu, 2008), increasing the LPS or TNF-α -induced $p22^{phox}$ gene expression in human aortic endothelial cells. The finding that NADPH oxidase activity is impaired in iron-deficiency and is corrected with iron supplementation (Kurtoglu et al., 2003) strengthens the correlation between intracellular iron and NADPH oxidase activity proposed here.

An increase in the expression of CD71 indicates cellular necessity for iron acquisition. This transferrin receptor expression is usually seen in activated and/or proliferating cells. Interestingly, on the second week of the LHTL training camp (TDay 3 and TDay4), where training load was significantly higher than the first week (Figure 6-1a) there was increase in the expression of CD71 on neutrophils in post-training samples (Figure 6-3e). Such a finding may indicate the increased cellular requirement for iron usually seen in immature neutrophils, which are known to be released from the bone marrow into the circulation post-exercise (i.e. left-shift). Curiously, in samples obtained on TDay2, 3, and 4 after a minimum 12-hour rest (pre-training samples - excluding acute effect of exercise) the percentage of CD71 positive neutrophils was significantly lower than that obtained on TDay1. Such decline may indicate the exit of immature neutrophils or neutrophils whose physiological apoptosis had been delayed, mainly through post-exercise increase in circulating cytokines and GCS-F from circulation onto tissues.

Relative Hb_{mass} values were increased after the altitude camp, confirming the effectiveness of the protocol adopted in eliciting haematological changes (Table 6-2). Further, the demands of each training day were reflected in iron status as serum iron declined after one training day (TDay2). Such a finding is in consonance with the acute phase reaction model of iron-withdrawal by the RES system post-exercise, mostly through the increase in IL-6-triggered hepcidin release (Bode et al., 2012; Kemna et al., 2005; Nemeth et al., 2004). Previous analysis from our group (Chapter 5 in this document) and others (Badenhorst et al., 2014; McClung et al., 2013; Peeling et al., 2014; Robson-

Ansley, Walshe, & Ward, 2011) shows that serum hepcidin concentration is increased post-exercise. Further, this study had hypothesised the possible effects of cumulative exercise through multiple sessions through consecutive training days on hepcidin concentration. No significant differences on serum hepcidin concentration were found between samples obtained at the end of TDay2 (6.2±1.0 nM) and TDay3 (6.3±0.7 nM). Similarly, Peeling et al. (2009a) did not observe cumulative effects of two running sessions' 12-h apart in urinary hepcidin concentration. However, using a longer duration and intensity than the protocol adopted by Peeling et al. (2009a), Roecker et al. (2005) demonstrated that urinary hepcidin concentration was still elevated 24 hours after the cessation of the exercise. Despite a reported high correlation between urinary and serum hepcidin (r=0.82, Ganz et al., 2008), further investigation is required to determine hepcidin kinetics in serum and additionally, its kinetics with multiple exercise bouts in during consecutive training days, thus reflecting the training schedule of an elite athlete. It is important to highlight that one athlete had a 33% increase in hepcidin concentration from TDay 2 to TDay 3, which may be indicative of the athlete being a hepcidin responder as identified previously (Chapter 5).

One of the most significant challenges to the body when exposed to altitude, particularly with the addition of the training stress, is maintaining functionally available iron and iron stores - which are fundamental for cell proliferation and function. This study has shown that the haematological changes elicited by LHTL are not limited to erythrocytes. Phenotypical redistribution and functional alterations in leucocytes, demonstrated here by change in surface antigen expression and specific neutrophilic functions, have highlighted the importance of a balance between hypoxic exposure and physical demands. Hepcidin is a fundamental link between iron status, exercise-induced inflammation, and hypoxia (Peeling et al., 2008). While decreased iron stores and hypoxia down-regulate hepcidin transcription, exercise and inflammation causes its up-regulation. While the molecular mechanisms of such opposing effects described in literature were not analysed here, it is interesting to hypothesize that they may come to attenuate the acute phase reaction-induced iron sequestration, provided that the athletes maintain their iron supplement intake, hence preserving iron stores (ferritin) levels. Maintaining functionally available iron and iron stores throughout a LHTL training camp may come to promote immune surveillance of athletes, highlighted in this study by maintenance of the resting phagocytic capacity of neutrophils throughout the training camp and enhanced stimulated oxidative capacity of neutrophils at the end of the LHTL training camp.

Supplement II – Chapter 6

Table A – 2-week training schedule

	8-Jun Mon	9-Jun Tue	10-Jun Wed	11-Jun Thurs	12-Jun Fri	13-Jun Sat	14-Jun Su
AM1	Training session home base 8-10km Aerobic T2/T3	15min bike T2/T3 K4 session skills and drills/resistance/ change of pace work -- inc. 3 standing starts	K4 -- 6x300m rolling @ 100sr paddle backs on 10min base Target time set.	(4x6min @ T4(AT)) 10min base -- measure distance and Lactates 15min Bike after session T2	K1 - Speed endurance/lactate set. 2 (6x50sec off/10sec on -4min rest -4x45sec off/15sec on -4min rest- 3x40sec off/20sec on) 7min rest between sets. 15min Bike after session T2	K4 -- 1x500m time trial (race plan) - following TT K4 3(100m/200/100/200/ 100/200/100/200/ 100) 100m as on's 200m as off's 10min base.	Rest
AM2		K1 -- 8--10km wash leads in pairs (T3 on lead)		Rest - Massage	K4 / mixed K2's 5xbroken 750's as 250m T3/T2/T3	Rest	Rest
PM	Gym + 15min bike T2/T3	K1 two groups 8x1000m @ T3 on 7min base (2min between groups)	Gym + 15min bike T2/T3	Rest - Massage	Gym + 15min bike T2/T3	Rest	Rest

Table A – continued

	15-Jun Mon	16-Jun Tue	17-Jun Wed	18-Jun Thurs	19-Jun Fri	20-Jun Sat	21-Jun Su
AM1	Warm up + bike K4 session 15--20min skills and drills /resistance + 3 starts 3DS + 2x broken 500m as 200/100 off/100/100 off/200m (K2 3x3ds starts 6x2min 3mr @95sr 8x1min 105sr 2mr)	Warm up + bike – K4 -- 4 x broken 500m (sub max 90--95%) working on race plan (250m on/off/on) (K2 -- 6xsplit 750m's)	Warm up + bike – K4/K2 -- 5x300m rolling @ 105sr paddle backs on 12min base Target time set.	K1 2x8min T2, 4x6min T2, 5x3min T3 (T2 efforts 1min rest, T3 efforts 2 min rest)	Warm up + bike K4 -- 6x200m alt. race simulation 1st and last 200m of race (MINI MAX K4/K2). (K2 6x(50m/50m/100m) on 10min base)	Warm up + bike	Rest
AM2	K1 -- 9km in pairs done as 2km T2/1km T3	6km K1 -- T1--T2 recovery K1 paddle		Rest - Massage	4-6km K1 T1/T2 recovery	K4/K2 -- 3x500m time trials (race plans) on a 20min base first effort Finish with K1 paddle 2x4min, 6x2min all T2 1 min rest	Rest
PM	Gym + 20 min bike T2/T3	K1 two groups 6x1500m @ T3 on 9min base (2min between groups) 20min bike after session	Gym + 20 min bike T2/T3 or on-water resistance session	Rest - Massage	Gym + 20 min bike T2/T3	Rest	Rest

Table B – Resting daily variation (pre-training) of the expression of granulocytic phenotypes

		Mean	SD	% Change from Day 1	*p* (from TDay1)	% Change from previous testing day	*p* (previous testing day✧)
CD11b MedianFI (AU)	TDay 1	12778	1023				
	TDay 2	6293	945	-50.7	<0.001*	-50.7	0.002
	TDay 3	8153	1777	-36.2	<0.001*	29.5	0.715
	TDay 4	8612	1753	-32.6	0.001*	5.6	1.000
CD16 MedianFI (AU)	TDay 1	11077	2488				
	TDay 2	9179	1748	-17.1	0.015	-17.1	0.089
	TDay 3	10196	1744	-8.0	0.114	11.1	0.789
	TDay 4	10826	3161	-2.3	0.648	6.2	1.000
CD18 MedianFI (AU)	TDay 1	4391	428				
	TDay 2	2414	169	-45.0	<0.001*	-45.0	0.001
	TDay 3	2539	568	-42.2	<0.001*	5.2	1.000
	TDay 4	2941	470	-33.0	<0.001*	15.8	1.000
CD66b MedianFI (AU)	TDay 1	4256	618				
	TDay 2	2257	256	-47.0	0.001*	-47.0	0.006
	TDay 3	2605	290	-38.8	<0.001*	15.4	1.000
	TDay 4	2774	826	-34.8	0.001*	6.5	1.000
CD71 MedianFI (AU)	TDay 1	891	215				
	TDay 2	491	100	-44.9	0.002*	-44.9	0.015
	TDay 3	690	174	-22.6	0.052	40.5	0.399
	TDay 4	548	139	-38.5	0.003*	-20.6	1.000

✧Bonferroni adjustment for multiple comparisons.
*Significant differences from baseline values ($p<0.01$)

Table C – Correlation between iron and immune markers

		S_Iron	Tf	UIBC	TIBC	TSAT	CD18	CD16	CD66b	CD11b	CD71
S_Iron	Pearson Correlation		0.116	-0.591	0.008	**.965****	-0.527	0.004	-0.342	-0.435	-0.323
	Sig. (2-tailed)		0.853	0.294	0.989	**0.008**	0.362	0.995	0.573	0.465	0.596
Tf	Pearson Correlation	**.896***		0.711	**.967****	-0.124	0.349	0.089	0.207	0.311	0.057
	Sig. (2-tailed)	**0.04**		0.178	**0.007**	0.843	0.565	0.887	0.739	0.611	0.928
UIBC	Pearson Correlation	0.395	0.727		0.802	-0.777	0.737	0.252	0.562	0.689	0.436
	Sig. (2-tailed)	0.511	0.164		0.103	0.122	0.155	0.683	0.325	0.198	0.463
TIBC	Pearson Correlation	0.864	**.978****	0.803		-0.249	0.523	0.315	0.443	0.533	0.301
	Sig. (2-tailed)	0.059	**0.004**	0.102		0.687	0.365	0.605	0.456	0.355	0.622
TSAT	Pearson Correlation	0.854	0.537	-0.124	0.486		-0.67	-0.126	-0.487	-0.594	-0.436
	Sig. (2-tailed)	0.065	0.351	0.842	0.407		0.216	0.839	0.405	0.291	0.463
CD18	Pearson Correlation	0.701	0.498	-0.08	0.411	0.772		0.658	0.877	**.934***	0.83
	Sig. (2-tailed)	0.187	0.394	0.899	0.492	0.126		0.228	0.051	**0.02**	0.082
CD16	Pearson Correlation	-0.422	-0.102	0.595	0.052	-0.731	-0.576		**.920***	0.844	**.941***
	Sig. (2-tailed)	0.479	0.871	0.29	0.934	0.16	0.31		**0.027**	0.072	**0.017**
CD66b	Pearson Correlation	-0.313	-0.28	0.016	-0.194	-0.298	0.176	0.51		**.985****	**.987****
	Sig. (2-tailed)	0.608	0.648	0.979	0.755	0.626	0.777	0.38		**0.002**	**0.002**
CD11b	Pearson Correlation	-0.026	-0.182	-0.375	-0.222	0.174	0.669	-0.177	0.744		**.950***
	Sig. (2-tailed)	0.966	0.77	0.534	0.719	0.779	0.217	0.775	0.15		**0.013**
CD71	Pearson Correlation	-0.414	-0.678	-0.801	-0.706	0.014	0.289	-0.262	0.572	0.809	
	Sig. (2-tailed)	0.489	0.208	0.104	0.182	0.982	0.637	0.67	0.313	0.097	

Correlation between iron and immune parameters pre- (blue) and post-training (white) TDay2. For all correlations, n=5. Correlation is significant at p=0.05 (*) and significant at p=0.01 (**).

Chapter 7

Effects of exercise and training on immunological and iron related parameters in elite female kayak athletes during a period of high-volume training

Introduction

Research has demonstrated throughout various sporting modalities such as cycling, swimming and rowing, that athletes spend more than 75% of their training at low-intensity (lactate $\leq$ 2mM) endurance exercises (Laursen, 2010; Seiler & Kjerland, 2006), despite competing at supra-maximal intensities. Accumulating hours in these low-intensity training zones improves cardiorespiratory and local muscle fitness via tissue remodelling and substrate utilisation through increased mitochondrial mass and oxidative enzyme activity (Baar, 2006; Burgomaster et al., 2008; Hood, Irrcher, Ljubicic, & Joseph, 2006; Terada et al., 2002). In training phases where elite athletes are exercising at moderate intensities, training volume is usually increased. The physiological adaptations gained from a low-intensity HV training phase are different to those obtained from a low-volume HI training one (Ingham et al., 2008; Laursen, 2010), described previously in the literature review and in chapter five of this thesis. Further, the type of training activates distinct molecular signalling, where, HV training has been associated with calcium-calmodulin kinase pathway, and HI training with the AMP-dependent protein kinase pathway (Coffey & Hawley, 2007; Gibala et al., 2006). It has been suggested that HV training (60 or 120 minutes below VT1) may elicit enough molecular signalling to provide the expected performance improvements with less disturbance to the autonomic nervous system than a HI training period (Seiler et al., 2007). However, adaptations in immune function and iron metabolism following HV training periods are not yet well established.

The immune response to varying exercise intensities is well documented. However, most conclusions arise from single bouts of effort. Conclusions drawn from such studies may not be applicable to periods of training. Only a few studies have been found to describe immunological function throughout a period of decreased intensity and increased volume (Brown et al., 2015; Hack, Strobel, Weiss, & Weicker, 1994; Nieman, 2000). Comparisons of such findings to periods of increased training intensity in the same athletic population are still scarce. It may be postulated that by decreasing intensity the exercise-induced acute response may not be of the same magnitude and consequently may not illicit the same alterations in immune function as a HI period. According to the "J" curve model proposed by Neiman (1994), it would be expected that decreasing training intensity from 'high' to 'moderate' (~60% $\dot{V}O_{2max}$) would promote immuno competence. Syu et al. (2012) suggested that in healthy untrained subjects, chronic moderate exercise (cycling 30 minutes/day for 4 weeks at 60% of $workload_{max}$) may promote a glycolysis-

dominating shift in neutrophilic metabolism, thus enhancing chemotaxis, phagocytosis, citrate synthase activity and mitochondrial membrane potential. It has been suggested that a minimum exercise intensity of ~60% $\dot{V}O_{2max}$ is necessary for significant increases in plasma catecholamines (Viru, 1985). In a highly-cited study, Robson et al. (1999) demonstrated that long-duration (>3 hr) moderate exercise (55% $\dot{V}O_{2max}$) elicited a greater post-exercise neutrophilia than a HI (80% $\dot{V}O_{2max}$) exercise performed by athletes for one hour. The increased concentration of neutrophils in the circulation after the moderate-intensity trial remained more elevated five hours after the cessation of the exercise than that following the HI trial and only returned to baseline 24 hours post-exercise. Further, this same group demonstrated that while neutrophils were activated immediately post-exercise by both trials (measured through elastase release), LPS-stimulated oxidative burst capacity after both exercise protocols was decreased (Robson et al. 1999). Curiously, while neutrophilic degranulation returned to pre-exercise values after the HI trial, this function was still below baseline values 24-hours post moderate-intensity exercise. Despite the above mentioned and many other studies attempting to determine the effects of exercise intensity and volume on neutrophilic functions, definite conclusions are made difficult, as indicated by Peake (2002), due to the variety of individual training status, blood-sampling time points, exercise protocols and assay techniques employed.

A two-week training phase in which training volume increased by ~40% did not alter the resting distribution of circulating components of the T-lymphocyte subset ($CD3^{+}$) in female soccer players (Brown et al., 2015). Indeed, Robson (1999) demonstrated that post-exercise lymphocytosis was of greater magnitude after a HI (80% $\dot{V}O_{2max}$) compared to moderate intensity (55% $\dot{V}O_{2max}$) exercise bout. However, when analysing immune competence in elite swimmers throughout a training season, Rama et al. (2013) found an increase in reported URTI symptoms after a HV training phase, which, not coincidentally, was associated with a significant decrease in NK lymphocytes. More specifically, increased training volumes caused a redistribution of NK lymphocyte subsets in peripheral circulation, decreasing the more cytotoxic NK lymphocyte subset ($CD56^{dim}$) while increasing the more cytokine-producing $CD56^{bright}$ subset (Cooper, Fehniger, & Caligiuri, 2001; Poli et al., 2009; Rama et al., 2013). It is possible that the increase in circulating cytokines produced by the $CD56^{bright}$ subset is a mechanism aiming to augment the pro-inflammatory response, while recruiting other leucocyte subpopulations.

Vital for immune competence, iron status parameters have also been shown to vary according to the intensity of the training stimulus. Lehmann et al. (1997) described significant decreases in serum iron (23±4 to 16±6 μmol/L) and serum ferritin (71±40 to 28±19 mg/L) of elite distance runners exposed to a four-week HV training period consisting of a weekly average increase in training volume of 35% of baseline (85.9±14.2km week one and 174.6±26.7km week four). After a nine-week basic combat training (~72 km/week) serum ferritin was decreased by 20.1% and TSAT by 42.7%, reaching a functionally compromising transferrin saturation of 14.7+7.7% in female soldiers (McClung et al., 2009). This same group demonstrated a positive correlation between the decline in iron status, indicated by the increase in sTfR, and the decrease in running performance at the end of the combat training.

Clearly training characteristics of intensity and duration pose different stress to both immune and iron metabolism homeostasis. However, HV training periods employed frequently by elite athletes have not gained the deserved attention. Therefore, this study investigated the effects of a 10-day increased training volume camp in immune and iron-related parameters in elite female kayak athletes. It was hypothesized that while the HV training period would increase the concentration of leucocytes in circulation, it would decrease immune competency. Further, the increase in training volume during the HV camp was hypothesized to decrease resting iron status of elite female kayak athletes.

Methodology

Training and participants

The HV training camp was held from 14-24th of December 2015 (Table 7-1). In this camp a total of 153.2 kilometres on-water and 1266.2 minutes were recorded from each athlete, divided into training zones as shown in Table 7-2. As per previous camps, all training intervention was determined by AIS coaches with no intervention from the researchers. Four athletes were initially recruited to this training camp. One athlete was removed from training for medical reasons. The remaining three athletes (body mass=73.3+6.4 kg; age=23.7+4.7 years; $\sum 7_{skinfolds}$= 71.4+10.2 mm; peak $\dot{V}O_2$= 54.1+2.5 mL/kg/min) who participated in this study represented Australia in the Rio 2016 Olympics at the K1 500m and K2 500m events. Daily training load was considered the product of training duration

and session RPE, while cumulative training load was a rolling 7-day average of the daily training loads.

Table 7-1 – High-volume camp schedule

Date	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
	M	**T**	**W**	**Th**	**F**	**S**	**Su**	**M**	**T**	**W**	**Th**
GC Training Camp											
Athlete Arrival/Departure											
Capillary samples (Immune parameters)			TDay1		TDay2			TDay3		TDay4	
Capillary samples (Iron parameters)											

Schedule of training camp held in December 2015 at AIS Gold Coast training centre. Testing days (TDay) are later referred to in-text.

Blood Collection

Capillary blood collection was performed in accordance with the methodology described in this thesis for the previous training camps (Chapters 5 and 6). Briefly, pre- and post-training samples (300µL) were collected from each athlete after a 5-10 minute vasodilative cream (Finalgon®) was applied to their earlobe. Samples were immediately transported and analysed for immune phenotypes and neutrophilic phagocytic function and PMA-stimulated oxidative capacity, and previously described in the *Methodology* chapter of this thesis (Chapter 3). Remaining capillary blood was centrifuged (300 x g for 10 minutes) and serum was aliquoted (~100µL), stored at -80°C and later analysed for iron parameters as per the *Methodology* chapter of this thesis (Chapter 3).

Statistical Analysis

Maintaining the standard of statistical analysis performed throughout this study, statistical analysis of the daily variations from pre- to post-training was performed through paired *t*-test, despite the small sample size (n=3). De Winter (2013) has highlighted that while there is a greater probability of false positive results when adopting a small sample size there is no objection towards its use. Hence, the percent change from pre- to post-training for each of the parameters analysed were quantified per individual athlete, as suggested by Vaux (2012), and brought to the reader's attention when necessary (>20%). ES were calculated according to Cohen (1988) and considered 'small' $\leq$0.2, 'medium' =0.5 and 'large' $\geq$0.8. Based on the number of athletes, this study was considered a large case study

and data on each athlete were displayed in the figures. Combined analysis of pre-samples throughout the HV camp were made through linear mixed model, considering the effects of each training day. Models were selected based on lowest AIC and number of parameters. Residuals were explored and observed values were plotted against model-predicted values to assert model's goodness-of-fit.

Results

Training

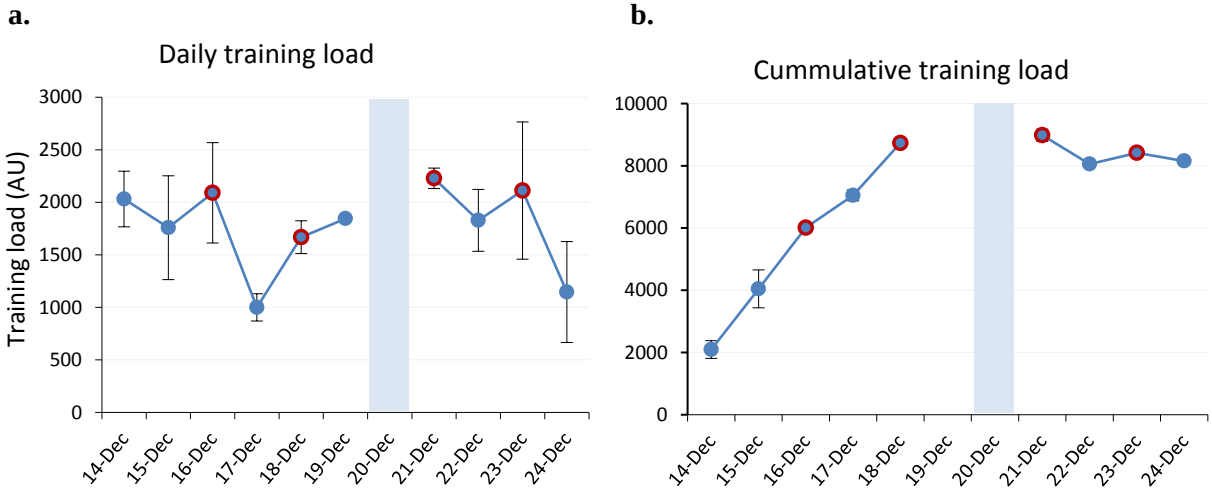
The HV camp accumulated a total of 153.2km 'on-water' over 787.6 minutes of training throughout a 10-day period. This training camp involved 480 minutes of 'off-water' training (considered T1) which included up- and down-hill running as well as strength and conditioning training. The allocation of time and distances per training zone is detailed in Table 2.

Table 7-1– Distance and time per training zone throughout the training camp

	T1	T2	T3	T4	T5	T6	T7	T8
Total Kilometres 'on-water'	0	94	33	7.2	7.6	9.9	1.1	0.4
Total Minutes	480	517.5	165	30.2	30.4	38.1	4	1

Training load variation throughout the camp is shown in Figure 7-1. TDay3 had the highest training demand with a training load of 2228±97.5 AU, followed closely by TDay 4 and TDay1 with 2111±653 AU and 2090±477 AU, respectively.

Figure 7-1 – Training load throughout the high-volume camp

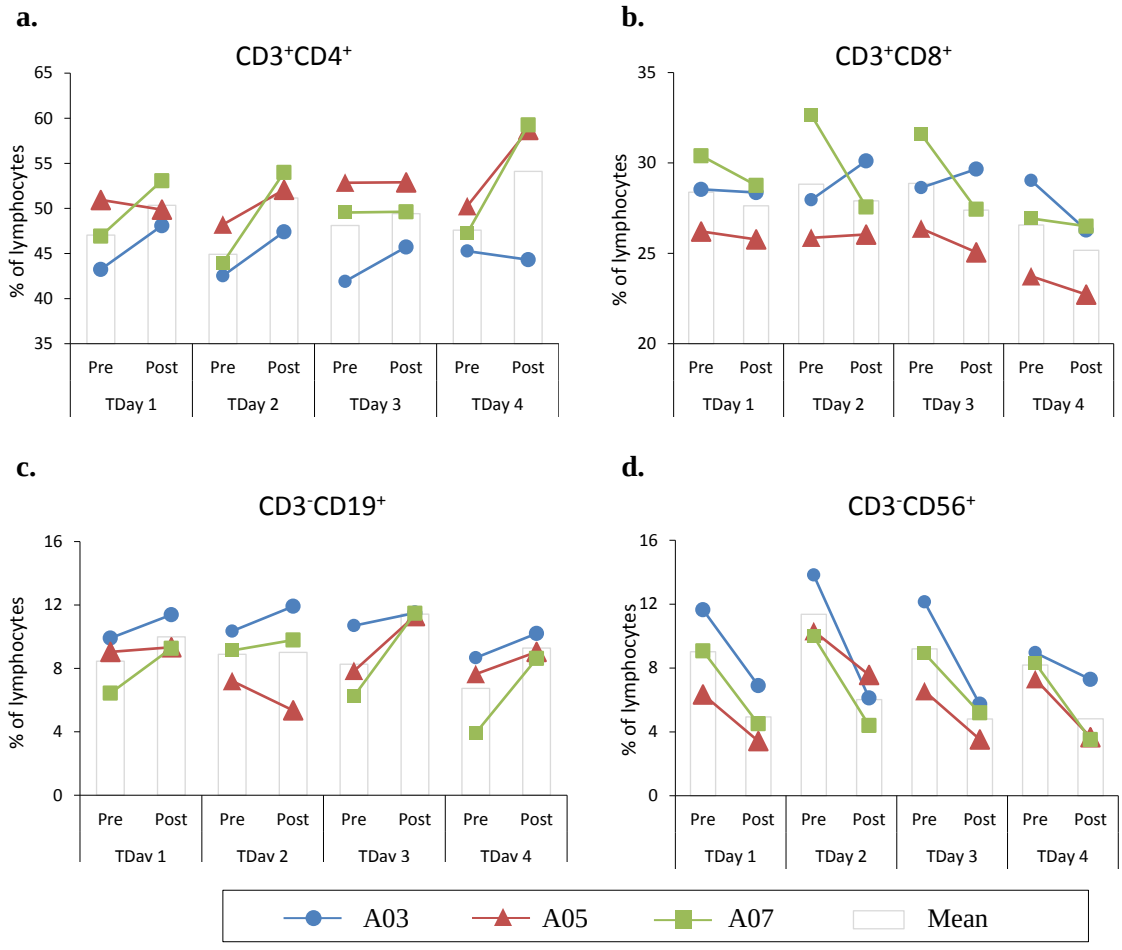


Acute Changes

Leucocyte phenotypical distribution and antigen expression

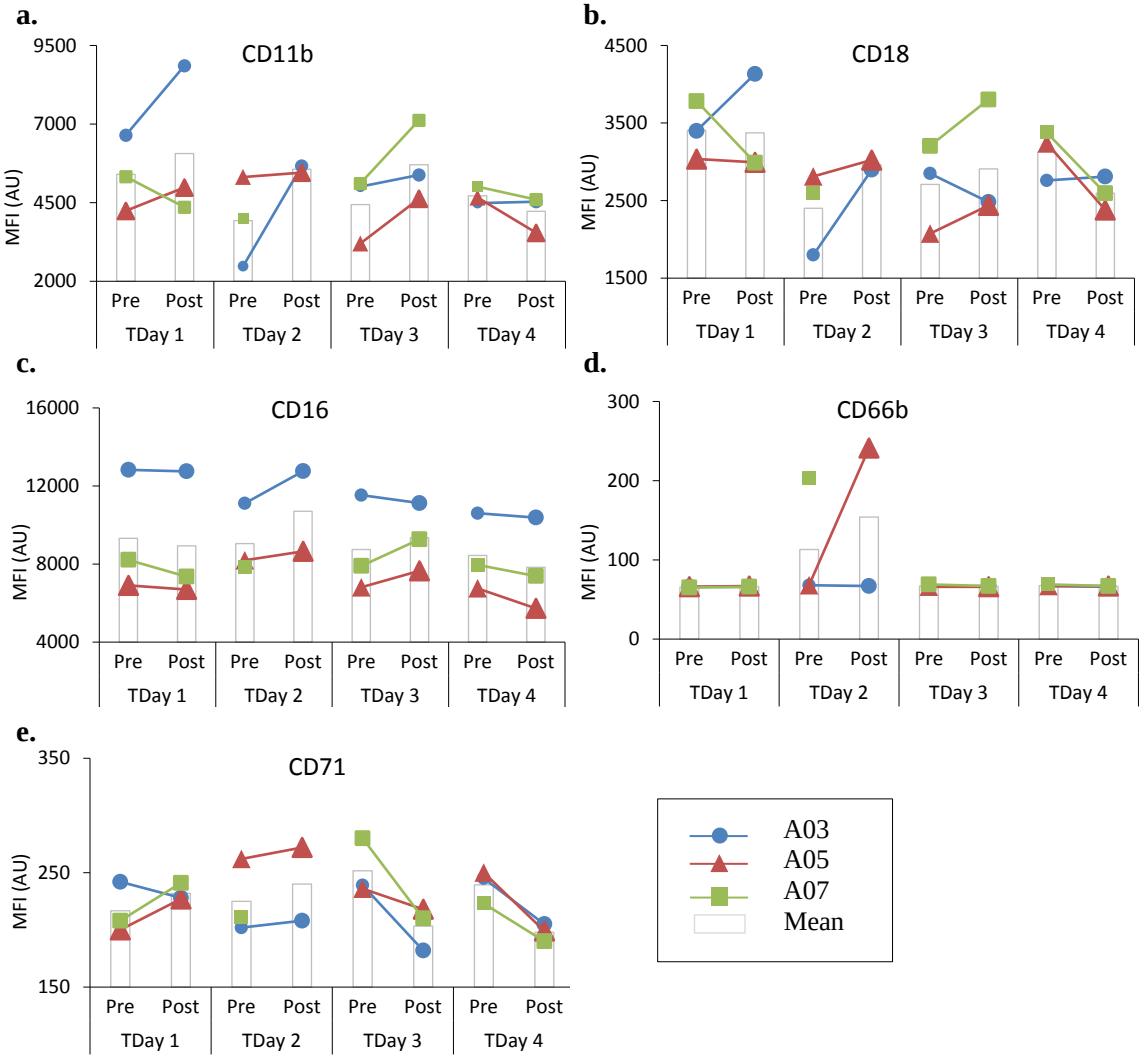
Distribution of circulating lymphocyte sub-populations from pre- to post-training was maintained throughout the HV camp (Figure 7-2). There were no significant changes in the proportions of circulating T-lymphocytes, $CD3^{+}CD4^{+}$ and $CD3^{+}CD8^{+}$, between pre- and post-training samples. There was a trend towards an increase in circulating percentage of B lymphocytes post-training (Figure 7-2c). Samples from athlete A07 showed the most accentuated changes from pre- to post-training in the distribution of circulating B lymphocytes ($CD3^{-}CD19^{+}$), with values increasing 1.8 and 2.2-fold on TDay3 and TDay4, respectively. Circulating distribution of NK lymphocytes ($CD3^{-}CD56^{+}$) was the lymphocyte sub-population most affected by training, with average declines >40% on every testing day (Figure 7-2d).

Figure 7-2 – Distribution of lymphocyte sub-populations pre- and post-training



Neutrophil surface antigen expression did not present significant variation from pre- to post-training (Figure 7-3). A mean increase in the expression of CD66b on circulating neutrophils from pre- to post-training (68±0.0 to 179.7±97.7 AU p=0.186) was observed on TDay2 (Figure 7-3d). Such increase was mainly attributed to the approximate 250% increase in the expression of CD66b in neutrophils obtained from one particular athlete (A05 Figure 3d). The expression of CD71 from pre- to post-exercise showed two distinct patterns throughout the camp (Figure 7-3e). On the first two testing days (first week of the HV camp) there was a trend towards an increase in CD71 expression post-training, with observed increases of ~15% for athletes A05 and A07. The second training week, however, there was an average 20% decrease from daily pre-training values in TDay3 and TDay4 (Figure 7-3e).

Figure 7-3 – Neutrophil phenotypical distribution pre- and post-training

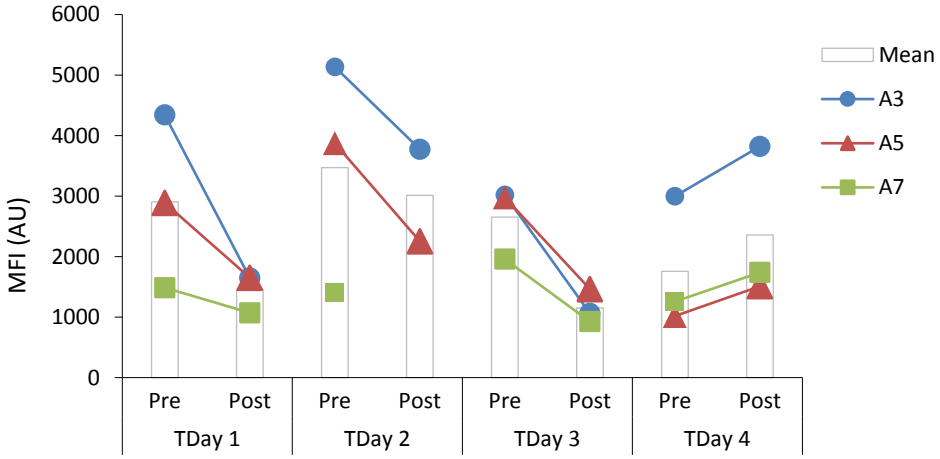


* Significant difference (p<0.01) between pre- and post-training
‡ Significant difference (p<0.05) between pre-training values and baseline (Pre-training TDay 1)
TDay 2 post-training sample from athlete A07 was insufficient.

Granulocyte functions

There was a trend towards decreased phagocytic capacity from pre- to post-training (Figure 7-4). The two most marked decreases occurred on TDay1 (2905±1428 AU to 1455±333 AU p=0.162 ES=1.6) and TDay3 (2562±601 AU to 1150±279 AU p=0.03 ES=3.4) with average post-training values 49% and 57% lower than pre-exercise values, respectively. Despite showing the highest pre-training phagocytic capacity (4504±892 AU), TDay2 was also marked by a >33% training-induced decline in phagocytic capacity (p=0.055, ES=1.5). Contrary to the previous testing days, TDay4 was marked by an increase in phagocytic capacity of 34% from pre- to post-training values (1757±1081 AU to 2357±1273 AU p=0.033 ES=0.5).

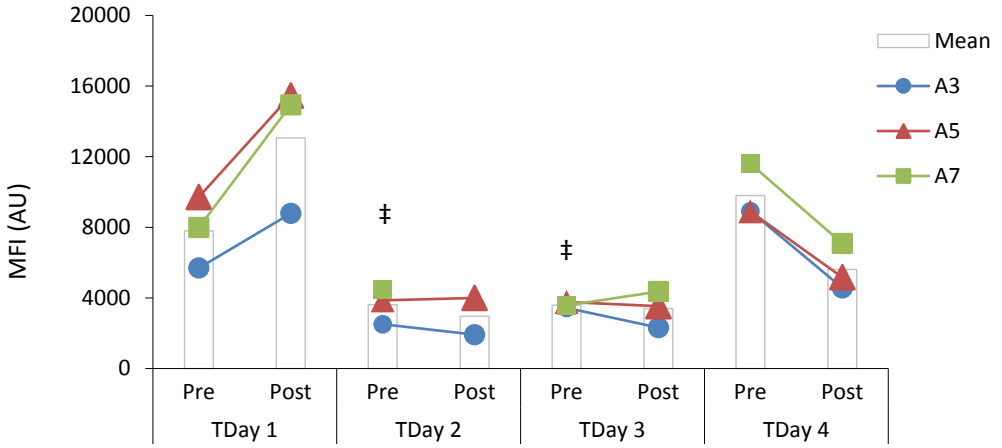
Figure 7-4 – Phagocytic capacity of granulocytes pre- and post-training.



* Significant difference (p<0.01) between pre- and post-training
‡ Significant difference (p<0.05) between pre-training values and baseline (Pre-training TDay 1)

TDay1 was marked by a training-induced increase in the capacity of PMA-stimulated peripheral blood granulocytes to produce ROS (assessed via MFI) from 7800±2016 AU pre-training to 13060±3711 AU post-training (p=0.044 ES=1.8) (Figure 5). On this day, athlete A07 had an 87% increase in stimulated ROS production, while the other two athletes had increases of over 50% of pre-training values. This trend was not observed in the subsequent testing days with values declining from pre- to post-training, reaching statistical significance on TDay4, where mean values declined from 9802±1560 AU pre-training to 5608±1318 AU post-training (p=0.003 ES=2.9).

Figure 7-5 – Stimulated oxidative burst capacity of granulocytes pre- and post-training.



* Significant difference (p<0.01) between pre- and post-training
‡ Significant difference (p<0.05) between pre-training values and baseline (Pre-training TDay 1)

Iron Studies

Table 7-3 shows the effects of one day of increased volume-training on iron parameters. After haemovolumetric corrections (mean plasma volume change = -3.9±12.6%) (Dill and Costill, 1974), post-training values demonstrate the increase in serum iron and consequently, transferrin saturation. There was a ~20% decline in transferrin post-training and a marked decrease in both unsaturated and total iron binding capacity.

Table 7-2 – Iron parameters pre- and post-training on TDay2

	Pre-training			**Post-training**			**Mean % Change**	**t**	***p***	**ES**
	Mean		**SD**	**Mean**		**SD**				
SI (µmol/L)	7.9	±	7.8	13.8	±	1.6	220.4	-1.342	0.408	1.3
Tf (µmol/L)	33.6	±	4.6	27.0	±	2.2	-18.6	1.384	0.398	-2.0
UIBC (µmol/L)	51.8	±	14.0	32.4	±	5.6	-33.6	1.400	0.395	-2.0
TIBC (µmol/L)	59.7	±	6.2	46.2	±	7.2	-21.6	1.421	0.390	-2.0
TSAT (%)	14.0	±	14.5	30.0	±	1.3	373.1	-1.439	0.387	-2.0

It is important to highlight that athlete A05 presented pre-training values for serum iron of 2.4 µmol/L, below the current reference range established by AIS of 3-36 µmol/L for female athletes. Such low concentration increased the standard deviation and possibly overestimated the mean percent change. If such measurement was not considered, there would still be an average 11% increase in serum iron post-training. TSAT is calculated from iron values (SI/TIBC), therefore, if the values obtained from athlete A05 were to be disregarded, the change from pre- to post-training would be of 20%. Athlete A05 UIBC

values were above the reference range (13-56 µmol/L) with pre-training value of 61.7 µmol/L.

Daily Variations

Leucocyte phenotypical distribution and surface antigen expression

Neutrophilic distribution in peripheral blood amongst the athletes did not vary significantly between the pre-training samples collected during the HV training camp. However, individual (per athlete) variations in circulating neutrophilic phenotypes were observed. Compared to baseline, on TDay2, circulating neutrophils from athlete A03 had a 63% decrease in the expression of CD11b, a 47% decline in CD18 and a 16.5% decline in expression of CD71. The expression of these markers was still decreased on TDay3 and CD11b and CD18 remained 32.5% and 19% lower than baseline values on TDay4. On TDay 3, samples from athlete A05 were marked by a 24.5% and 32% decline from baseline values on the neutrophilic expression of CD11b and CD18, respectively. Increases from baseline values in the expression of CD71 in neutrophils obtained from A05 were observed in TDay2 (31%), TDay3 (18%) and TDay4 (25%). Athlete A07 showed similar pattern in increased CD71 expression with highest increase from baseline observed on TDay3 (35%). While there was a paucity in the expression of CD66b on neutrophils from athletes A03 and A05, athlete A07 had a 212% increase in the expression of this marker on TDay2, with values returning to baseline on TDay3 and TDay4. Neutrophilic expression of CD16 was decreased in samples obtained on TDay4 from all athletes, with the greatest change observed in athlete A03 with a 17% decrease from baseline.

Aiming to evade the acute effects of training on phagocytic capacity, samples obtained prior to every testing day were analysed through linear mixed model and demonstrated considerable variation amongst the athletes (Figure 7-4). Granulocytes from athletes A03 and A05 had an 18% and 34% increase in phagocytic capacity on TDay2, respectively, compared to their baseline (pre-training sample TDay1). However, as the camp progressed, granulocytes from all athletes showed a reduction in their capacity to ingest FITC-labelled *E. coli*. On TDay4, granulocytes from athlete A05 had a 65%, athlete A03 a 31% and A07 a 15% decline in phagocytic capacity compared to baseline.

Linear mixed modelling found a significant effect of day (p=0.006) in the stimulated oxidative burst capacity of granulocytes (Figure 7-5). Analysing granulocytes from pre-training samples, stimulated oxidative burst capacity on TDay2 (3613±1004 AU p=0.006) and TDay3 (3593±169 AU p=0.008) were significantly decreased from baseline (7800±2016 AU). By TDay4, however, oxidative capacity was restored (9802±1560 AU p=0.114).

Discussion

The HV training camp did not seem to cause significant redistribution of either lymphoid or myeloid lineages in circulation. However, significant individual variations in function-related granulocytic surface antigen expression, phagocytosis and oxidative burst capacity highlighted the need for individual monitoring.

Lymphocyte sub-populations demonstrated distinct patters of mobilization post-training. Lymphocyte mobilization has been well described in the literature comparing moderate (55% $\dot{V}O_{2max)}$ and high (80% $\dot{V}O_{2max}$) intensity bouts of exercise, with the latter having a greater impact on cellular redistribution (Nieman et al., 1994; Robson, Blannin, Walsh, Castell, et al., 1999). While the HV moderate-intensity training adopted in this camp did not seem to mobilize T lymphocyte subsets (Figure 7-2a and 7-2b), there was a tendency towards a post-training increase in circulating B lymphocytes distribution (Figure 7-2c). Post-exercise B lymphocyte mobilization has been previously described in response to a range of exercise intensities, with higher intensities producing greater B lymphocyte redistribution from bone marrow into circulation. Recently, the post-exercise increase in circulating B lymphocytes has been attributed to an increase in an immature sub-population of B lymphocytes ($CD3^{-}CD19^{+}CD27^{-}CD10^{+}$; Turner et al. 2016) . Utilising a rodent model, Engler et al. (2004) suggested that the redistribution of B lymphocytes from the bone marrow to the spleen would enable an immediate immune response after repeated stress events (experimental group was exposed to aggressive male C57BL/6 mice). Despite the increase in circulating percentage of NK lymphocytes at the onset of exercise, many have reported declines of up to 40% in this population in recovery stages (from 5 minutes' post-exercise) after prolonged exercise (~120 minutes) (Gabriel & Kindermann, 1997; Shek et al., 1995). The strain imposed by the non-kayak training modalities adopted during this camp (i.e. down-hill running) may have contributed to the

decline in the percentage of NK lymphocytes in circulation of similar magnitude to the above mentioned studies. NK lymphocyte redistribution post-exercise has been attributed to this population's function in muscle tissue repair, which would explain their decrease from peripheral circulation (Malm et al., 2000).

Throughout the HV training camp, the expression of specific antigens on the granulocytes' surface was minimally altered. In the first week of training, there was a trend towards a mean increase in iron requirement of granulocytes post-training, measured by the increase in CD71 expression. However, on the second week of the camp post-training expression of CD71 on granulocytes presented decreases of up to 25% of pre-training values (Figure 3e). Information on the relationship between CD71 expression in granulocytes and their function is scarce. In granulocytes, CD71 is expressed in bone-marrow derived normal blasts, with highest expression observed in pathological conditions such as acute-leukemic granulocytes due to their over-proliferation in the bone-marrow (Liu et al., 2014). In resting circulating granulocytes CD71 is minimally expressed (Dunphy, 2010). To date, only Zarco et al. (1999) have demonstrated an increase in CD71 expression on circulating granulocytes from healthy donors after G-CSF administration. The increased expression of genes coding for components of the NADPH oxidase ($p22^{phox}$, $p47^{phox}$, $p67^{phox}$, $gp91^{phox}$) in peripheral neutrophils stimulated with opsonized *S. aureus* and *E. coli* LPS was demonstrated (Matthews et al. 2007). Iron is a regulator of gene expression, being present as Fe-S proteins in numerous DNA binding proteins (e.g. glycosylases; Boal et al., 2005; Porello, Cannon, & David, 1998), DNA processing, repairing and replicating enzymes (e.g. helicases and DNA polymerases; Pokharel and Campbell, 2012; Netz et al., 2011; Rudolf et al., 2006), RNA polymerase (in *M. acetivorans*; Jennings et al., 2015) , RNA-modifying enzymes (Kimura & Suzuki, 2015) and many others recently reviewed in Mettert and Kiley (2015) and Fuss et al. (2015). Thus, it is reasonable to suggest that the transferrin receptor would be upregulated in transcriptionally active granulocytes. Individual data from athlete A03 demonstrated the greatest training-induced decline in CD11b, CD18 and CD71 expression. Such declines in expression of CD11b/CD18 and CD71 remained below baseline values throughout the training camp. Interestingly, this athlete was the only one in the cohort to report flu-like symptoms (nasal congestion with productive cough) and fatigue on TDay1 (verbal communication with investigator) and was later prescribed oral antibiotics by a physician. Further studies should aim to determine CD71 kinetics in granulocyte and its possible correlation with pathogen extermination rates.

Daily HV training had a suppressive effect on neutrophilic phagocytosis of *E.coli*, with post-training declines observed in TDays 1, 2 and 3. In contrast, the samples collected at the end of TDay4, demonstrated enhanced phagocytic capacity compared to pre-TDay4. While the TDay4 post-training values may indicate possible restoration of phagocytic capacity as values tended to return to (but were still less than) baseline values (pre-TDay1), further investigation utilising more sampling time-points is required to ensure that the improvement is sustained post-camp. Interestingly, on TDay1, the stimulated oxidative burst capacity was increased from pre- to post-training. However, this trend was not sustained throughout the camp, with values usually remaining unchanged (TDays 2 and 3) before declining from pre- to post-training on TDay4. TDay2 was preceded by a low training load day and TDay3 by a rest day. Interestingly, these two days showed marked decline in stimulated oxidative burst capacity, compared to baseline. Mochida (2007) suggested that neutrophilic functions may compensate for themselves post-exercise. *In-vitro*, such observation might be valid, however, through a physiological perspective, in effective microbicidal killing, ROS production would follow a successful/complete phagocytosis (Briggs, Robinson, Karnovsky, & Karnovsky, 1986; Segal, 2005). Assembly of the NADPH oxidase complex however is not an exclusively post-phagocytic event, as molecular signalling originates from stimuli such as β_2-integrins and FcγRs receptor coupling (Anderson et al., 2010; Nathan, 1987), chemoattractants (e.g. fMLP; Dahlgren and Karlsson, 1999), cytokines (e.g. GM-CSF and TNF) and components of the complement cascade (Condliffe et al., 2005) which have been shown to promote NADPH oxidase ROS formation. Therefore, while *in-vitro* stimulated oxidative burst capacity may be enhanced post-training, it does not necessarily translate to increased immune competence of the athlete, as incomplete/frustrated phagocytosis may lead to ROS formation and release into extra-phagosomal space which could potentially harm the host.

The HV camp also brought about individual changes in iron metabolism. There was an increase in serum iron which might be reflective of the non-kayak-specific modalities (i.e. running) adopted in this camp. Such modalities have been reported to elicit foot-strike haemolysis (Miller, Pate, & Burgess, 1988; Telford et al., 2003). This concept acknowledges that the increase in serum iron after endurance demands might be caused by the mechanical trauma which leads to the breakage of the plasma membrane (i.e. haemoglobin degradation) of erythrocytes consequently releasing haem into circulation (Janakiraman, Shenoy, & Sandhu, 2011; Salvagno, Lippi, Tarperi, Guidi, & Schena, 2015;

Telford et al., 2003). Due to serum iron levels varying up to 50% throughout the day, a reliable iron status requires other iron-related parameters such as ferritin, transferrin and transferrin saturation to be measured (Scales, Vander, Brown, & Kluger, 1988). Circadian variation of iron demonstrates that serum iron levels are at their highest from 8:00-12:00 hours with declines thereafter, reaching its nadir between 16:00-20:00 hours. Blood collection in this study occurred from 5:30-6:30 hours prior to the first training session and at 16:00-17:00 hours after the completion of the last training session of the day. Scales et al. (1988) did not show significant differences in the plasma iron concentration at these two time-points, which indicates that the sampling time-points elected were not greatly influenced by the physiological diurnal variation in serum iron. Further, as diet was not controlled, it is possible that athlete A05 had an iron-rich meal throughout the day, possibly restoring the values of circulating iron from the below reference range value of 2.4 µmol/L obtained pre-training to 12.7 µmol/L post-training. Even so, dietary intake in the short time elapsed from pre- to post-samples do not seem sufficient to cause increases of such magnitude to serum iron as, using standardised meals, Ridefelt et al. (2010) did not find significant changes in serum iron in the blood samples obtained hourly, for 4 hours post-meals in healthy young men. It is known that in ID, the rate of iron absorption is increased, however full restoration of serum iron to adult reference-range values only occurs after days of iron replenishment (Frazer et al., 2002). Despite the possible confounding variables which may have influenced serum iron values, analysis of UIBC and Tf demonstrate the capacity of the HV training to cause disturbance to individual iron status, upholding the necessity for continuous monitoring of iron status, including training periods where training intensity is lowered.

This study demonstrates that a HV training camp has a moderate effect on immune modulation and iron metabolism. The most distinct alterations observed may be attributed to the adoption of non-kayak specific modalities, which would likely have caused increased muscle damage, brought about by eccentric contraction of large muscle mass (e.g. downhill running), however, further research is warranted (Peake et al., 2005; Pizza et al., 1995). Despite not having significantly influenced granulocytic phenotypical expression and distribution, the HV camp modulated effector functions of phagocytosis and oxidative burst. Interestingly, these functions seem to have had compensated for each other throughout the camp.

Chapter 8

Longitudinal analysis of haematological and biochemical parameters in elite female kayak athletes throughout an Olympic selection training year

Introduction

Multi-peak training years require athletes to perform at their utmost multiple times per year. In an Olympic selection year, winning World Championships and World Cups guarantees a place at the Olympic Games. Longitudinal analysis of the impact of such demands are scarce in literature, particularly when assessing immune function and iron status. Therefore, the aim of this chapter is to provide a longitudinal analysis of the impacts that the HI, LHTL and HV camps had on the immune function and iron status of the elite female kayak athletes.

Owing to the singularities of venous and capillary blood sampling sites, comprehensively explored in Study I of this thesis (Chapter 4), this chapter will discuss only findings from venous blood samples, obtained prior to and at the end of each training camp. This will enable a longitudinal analysis of the training year, highlighting the effects of the different training periods - HI (April), a LHTL (June) and a HV (December) - on iron and immune-related parameters. The reader is referred to chapters five (2-week HI camp n=7), six (ten day LHTL camp, n=5) and seven (10 day HV camp, n=3) of this thesis if a revision of the characteristics of each camp and their influence on iron metabolism and immunological modulation is required.

Methods

Details and characteristics of each training camp and participating athletes can be found in Study II, III and IV (Chapter 5, 6 and 7, respectively) of this thesis.

Blood collection

Resting venous blood samples were obtained prior to the onset and at the last day of each camp as athletes arrived at the AIS Gold Coast training centre between 6-9am. After a 10-minute rest in the seated position, venous blood samples (10 mL) were collected into two separate tubes: an EDTA-containing tube and a serum separator tube (SST) (Vacutainer BD BioSciences, New Jersey, US). A five-part differential full blood count (HmX, Beckman Coulter, California, US) was immediately performed on the sample containing EDTA as an anticoagulant. Samples in the SST were allowed to clot at room

temperature for one hour before being centrifuged at 2000 x g for 10 minutes. Serum was then aliquoted into 1 mL tubes (Eppendorf®, Hamburg, Germany) and frozen at -80°C. Serum samples from all camps were thawed once only and analysed at the same time after no longer than eight months' storage under the above mentioned conditions. Analysis of iron parameters (serum iron, transferrin, soluble transferrin receptor, ferritin and unsaturated iron-binding capacity (UIBC)) was performed on a Cobas Integra® 400 plus biochemical analyser (Roche Diagnostics, Basel, Switzerland) after all calibrators, controls and standards were performed (in duplicates). TIBC was calculated from the addition of serum iron and UIBC values. The percentage of transferrin saturation was calculated from the formula SI/TIBC x 100. Immune- and iron-related parameters (myeloperoxidase, lactoferrin, interleukin-6 and hepcidin) were analysed through commercially available ELISA kits (MPO, IL-6 and Hepcidin were purchased from R&D Systems, Minnesota, USA and LF from ABCAM, Cambridge, UK) described previously.

Statistical Analysis

Initial assessment of pre- and post-camp values for each individual camp was made through paired samples t-test, after all the data was explored and all assumptions were met. Significance was set at $\alpha<0.05$. Calculation of effect size was performed according to Cohen (1988) where the difference of the means of the two groups divided by the sum of their standard deviations divided by 2 (*d*) is considered 'small' $\leq$0.2, 'medium' =0.5 and 'large' $\geq$0.8.

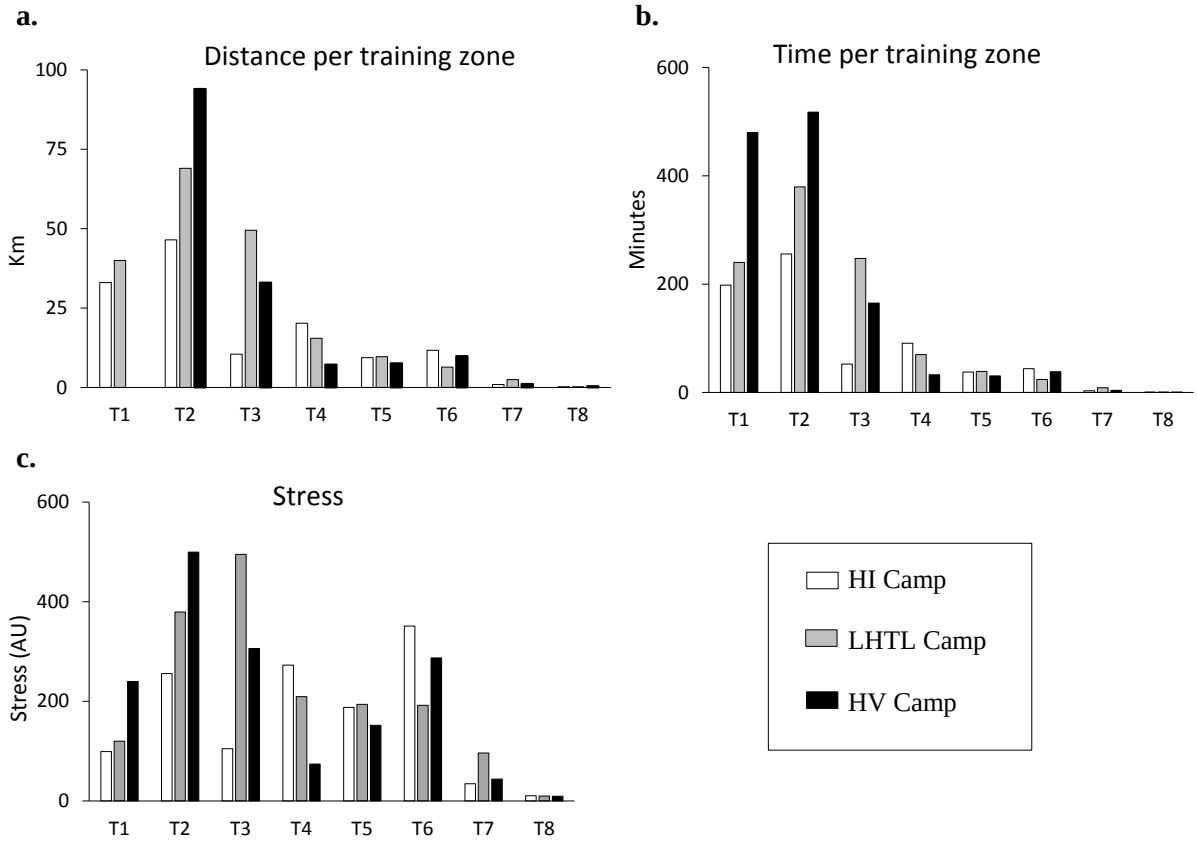
Combined analysis of iron- and immune-related parameters between the three camps was made utilizing a linear mixed model. Model selection was determined by lowest AIC and least amount of parameters, as previously described. Each outcome was modelled with fixed effects of time (pre- or post-camp), camp (HI, LHTL or HV) and interaction between these two effects (time*camp). The model controlled for random intercept and slope (time) for each athlete and camp was established as a repeated measure accounting for time of collection for each athlete (athlete*time). Estimated marginal means of fitted models with Bonferroni adjustment compared main effects of camp, time and camp*time. The goodness-of-fit of the models were established by exploring the residuals and by plotting observed values against model-predicted values.

Results

Training intensity and duration

Characterisation of each camp was based on the training distance ('on-water' kilometres), duration (minutes) and stress imposed per training zone. Figure 8-1 highlights the uniqueness of each camp according to these parameters. The HI camp, showed increased distance and time spent in training zone (T) 6, where athletes produced work at the 500m race pace, compared to the LHTL and HV camps (see Table 3-4 in methodology section for complete description of training zones). Adding to the altitude challenge, the LHTL camp was a highly demanding training period, demonstrated by the 2.5km spent in T7, 2.7-fold more than the HI camp, and the compiled distance and time spent on the aerobic zones (T2 and T3). As seen in Figure 8-1a during the LHTL camp the distance accumulated in T3 were 4.7-fold more than the HI camp. In the HV camp, the athletes worked for 94km in zone T2, twice as much as in the HI camp. Further, in the HV camp the athletes increased the time spent in other water-based training capacities, such as surf-ski and surfing (T1), as shown in Figure 8-1b.

Figure 8-1 – Duration and distance performed per zone in each training camp.

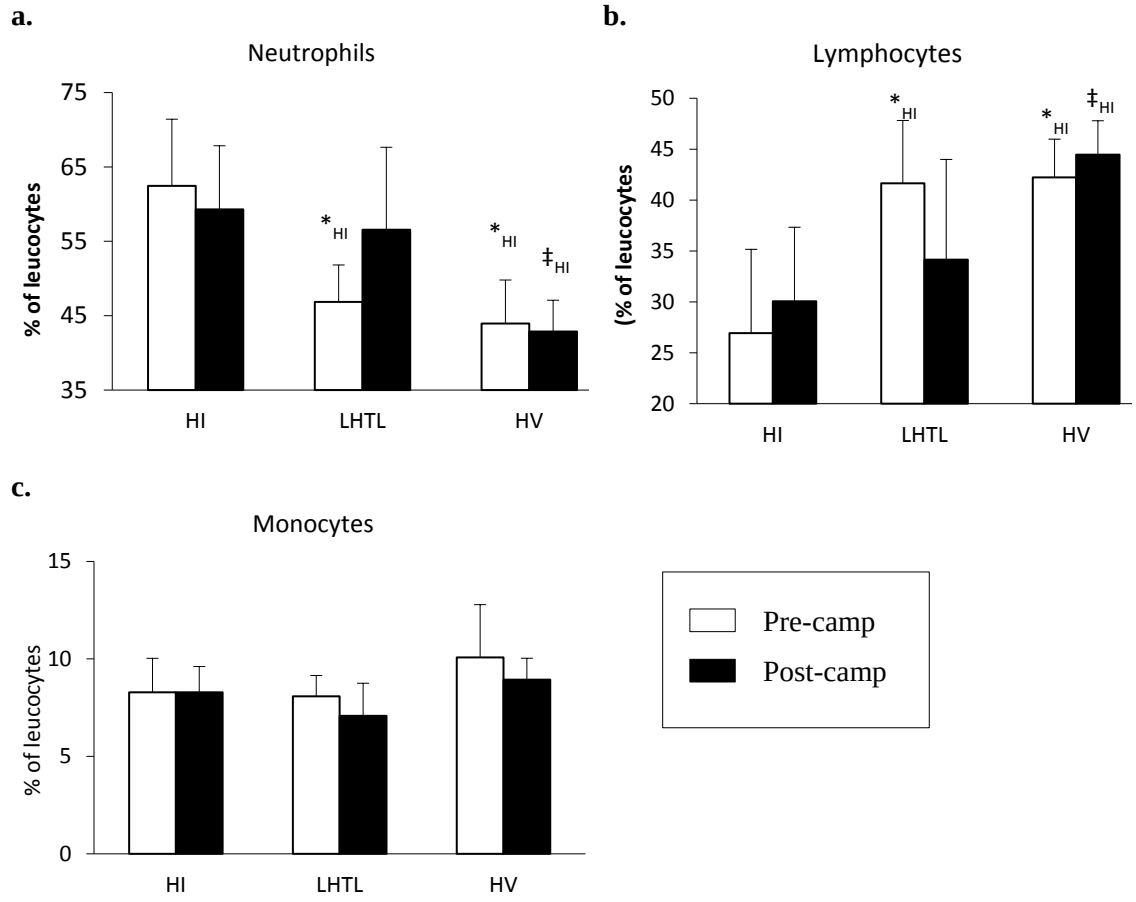


T=training zone. Refer to Table 3-4 in the *Methodology* chapter (3) for description of each training zone.

Blood-related parameters

Venous blood samples collected prior to and post-camps were employed in the analysis of the cumulative effects of the different training volumes and intensities adopted in each training period on iron and immune-related parameters. Each training camp caused a unique redistribution in circulating leucocyte sub-populations from pre- to post-camp (Figure 8-2a-c). In the LHTL camp there was an increase in the circulating percentage of neutrophils from pre (46.8±4.9%) to post-camp (56.6±11.1% p=0.076, ES=1.2), while there was an average 5% decrease from pre-camp values in both the HI and HV camps. Contrary to the neutrophilic population, in the LHTL camp, distribution of lymphocytes was decreased from pre- (41.6±6.1%) to post-camp (34.1±9.8% p=.121 ES=0.9), while a 11.6% and 6.8% increase from pre-camp values were observed in the HI and HV camps, respectively. While these values did not reach statistical significance, comparison of each time-point amongst the three camps through linear mixed model demonstrated a significant difference in the percentages of circulating leucocyte sub-populations throughout the training periods (Figure 8-2a-c). The average concentration of circulating neutrophils (pre-and post-camp) was significantly greater in the HI camp (60.9±8.6%) compared to both the LHTL (51.7±9.5% p=0.040) and HV (43.5±4.8% p<0.001) camp. Further investigation highlighted that the percentage of circulating neutrophils in the samples obtained prior to the LHTL (46.8±4.9% p=0.011) and HV (43.9±5.8% p=0.004) camps were ~25% lower than the values obtained prior to the HI camp (62.5±9.0%) (Figure 2a). At the termination of the camps, the difference between neutrophil concentration in the HI (59.3±8.5%) and HV (42.9±4.2%) camps remained significant (p=0.02) (Figure 8-2a). The percentage of circulating lymphocytes was significantly lower on the HI camp compared to both LHTL (p=0.022) and HV (p=0.001) (Figure 8-2b). Detailed investigation showed that samples obtained prior to the LHTL and HV camps were respectively 14% and 15% greater than the percentage of circulating lymphocytes obtained prior to the HI camp. As the concentration of circulating lymphocytes at the end of the LHTL camp decreased, no significant differences in these time points were observed between the HI and LHTL camps. However, values obtained after the completion of the HV camp were still 14% higher than those observed at the end of the HI camp. Percentage of circulating monocytes (Figure 8-2c), eosinophils and basophils (data not shown) did not differ significantly between data collection points.

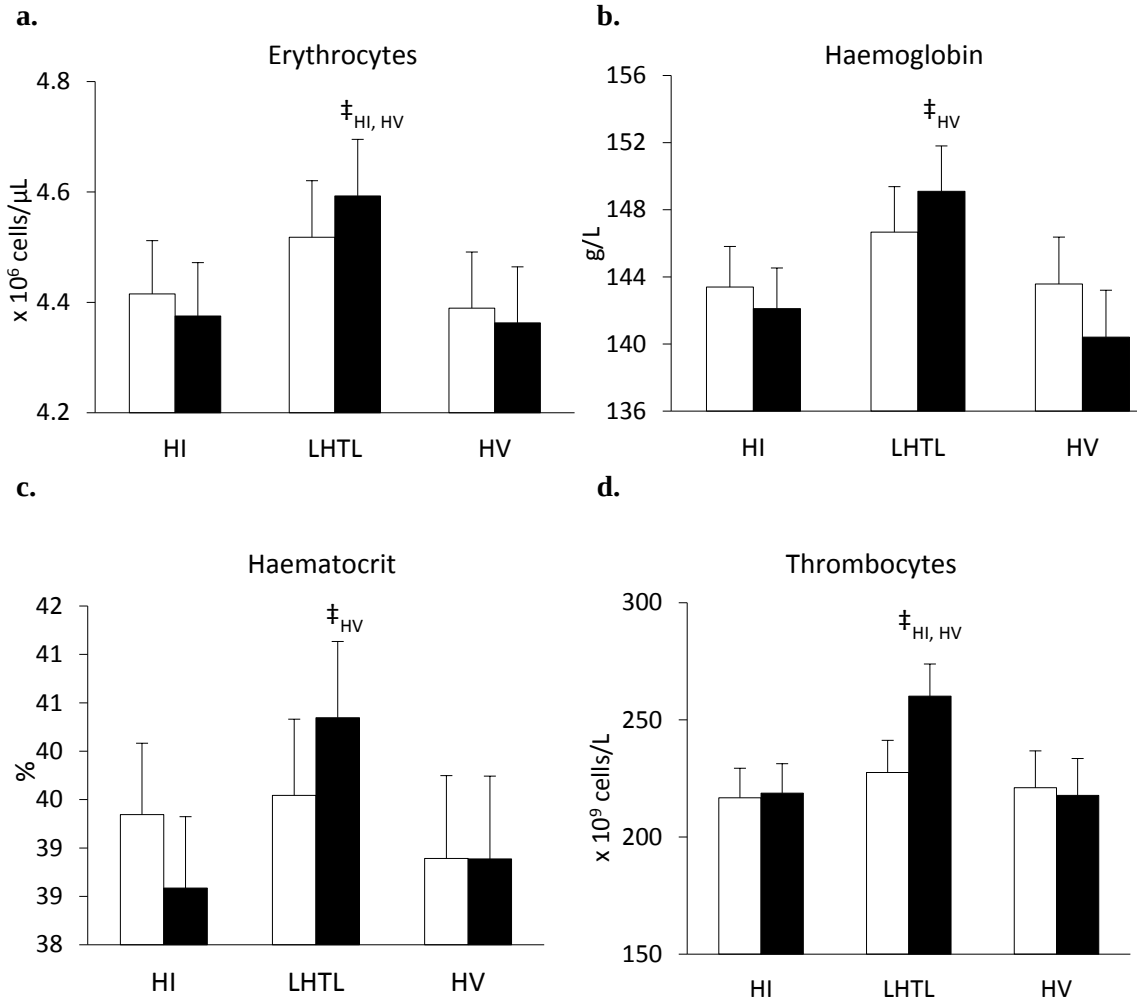
Figure 8-2 – Leucocyte populations pre- and post-camps



HI-high-intensity, LHTL live-high train-low, HV high-volume camp.
Solid bars represent means and error bars represent SD.
* Significant difference between pre-samples on assigned camps
‡ Significant difference between post-samples on assigned camps

Erythrocyte-related parameters varied throughout the different training camps. The highest values for all the erythrocyte-related parameters were obtained after the completion of the LHTL camp (Figure 8-3). At the end of the LHTL camp, erythrocyte concentration was 5.0% and 5.3% greater than that measured at the end of both the HI (p=0.04) and HV (p=0.035) camps (Figure 3a). At this same time-point, haemoglobin concentration was also 6.2% and 4.9% higher in samples obtained in the HV (p=0.032) and the HI camp (p=0.067), respectively (Figure 3b). Haematocrit at the end of the LHTL camp (40.3$\pm$0.8%) was significantly higher than at the end of the HI camp (38.8$\pm$0.9% p=0.035) (Figure 8-3c). Different to the HI and HV camps, there was a 12% increase in thrombocyte concentration at the end of LHTL camp from pre-camp values (p=0.056). Values for thrombocyte concentration at the end of the LHTL camp (260.1+13.7 x 10^9 cells/L) were significantly greater than at the end of the HI (218.7$\pm$12.6 x 10^9 cells/L p=0.004) and the HV camp (217.8$\pm$15.7 x 10^9 cells/L p=0.030).

Figure 8-3 – Erythrocyte-related parameters pre- and post-camps



Pre-camp (▭) Post-camp (▬)
HI-high-intensity, LHTL live-high train-low, HV high-volume camp.
Solid bars represent means and error bars represent SD.
* Significant difference between pre-samples on assigned camps
‡ Significant difference between post-samples on assigned camps

A comprehensive iron profile of the different training camps is provided in Table 1. At the end of the LHTL camp, serum iron was increased by 16.1% of pre-camp values, despite not reaching statistical significance. Transferrin saturation was reduced by 2% of pre-camp values in the HI camp, while an increase of 12.3% was observed at the end of the LHTL camp. While serum ferritin concentration was reduced at the end of the HI camp by 4.1%, iron stores were maintained throughout the LHTL and HV camps. Mean serum ferritin concentration obtained in the HI camp (pre- and post-camp) was approximately 30% less than that obtained in the HV camp (p=0.025). STfR-Ferritin index was significantly higher (p=0.025) in the HI camp, compared to the HV camp (Table 8-1).

Table 8-1– Iron parameters prior to and post training camps

	Camp	Pre-camp Mean	Pre-camp SD	Post-camp Mean	Post-camp SD	Mean % Change	t	*p*	ES
Serum Iron (µmol/L)	HI	20.9	6.6	18.9	3.6	1.0	0.731	0.492	0.39
	LHTL	18.1	2.5	21.0	7.0	16.1	-0.986	0.380	0.60
	HV	18.1	4.4	18.1	4.2	1.1	-0.025	0.982	0.01
UIBC (µmol/L)	HI	34.7	8.3	38.4	9.9	12.0	-1.123	0.304	0.40
	LHTL	33.9	7.8	33.3	10.6	-0.4	0.147	0.890	-0.06
	HV	38.2	11.5	35.9	10.0	-4.7	0.894	0.466	-0.21
TIBC (µmol/L)	HI	55.6	7.6	57.3	8.1	3.2	-1.213	0.271	0.22
	LHTL	52.0	8.5	54.3	10.8	4.3	-1.080	0.341	0.24
	HV	56.3	8.9	54.0	8.1	-3.8	1.402	0.296	-0.27
Transferrin (µmol/L)	HI	28.8	3.7	29.9	3.8	3.8	-1.959	0.098	0.28
	LHTL	27.3	4.6	28.8	6.3	4.9	-1.285	0.268	0.27
	HV	29.6	4.5	28.7	4.3	-3.1	2.200	0.159	-0.21
TSAT (%)	HI	37.6	11.4	33.8	9.4	-1.9	0.801	0.454	-0.36
	LHTL	35.2	5.4	38.9	12.3	12.3	-0.615	0.572	0.42
	HV	33.1	11.1	34.2	9.5	5.5	-0.332	0.772	0.11
Ferritin (pmol/L)	HI	152.5	43.9	147.4	59.9	-4.1	0.461	0.661	-0.10
	LHTL	180.1	70.9	174.7	58.3	-0.2	0.498	0.644	-0.08
	HV	215.4	76.0	209.9	66.5	-0.1	0.307	0.788	-0.08
sTfR (nmol/L)	HI	33.8	6.7	32.3	6.1	-4.2	2.236	0.067	-0.23
	LHTL	27.1	3.5	27.9	0.8	3.9	-0.551	0.611	0.34
	HV	28.7	3.9	27.2	1.3	-4.5	1.005	0.421	-0.57
sTfR-Ferritin Index	HI	1.6	0.3	1.5	0.4	1.5	0.828	0.439	0.09
	LHTL	1.2	0.2	1.3	0.1	1.3	-0.344	0.749	0.19
	HV	1.2	0.1	1.2	0.1	1.2	1.485	0.276	0.52

UIBC=unsaturated iron binding capacity TIBC=total iron binding capacity Tf=transferrin TSAT=transferrin saturation sTfR= serum transferrin receptor. Degrees of freedom for each camp: HI (6), LHTL (4), HV (2)

*Significant at $p<0.05$ HI (high-intensity), LHTL ("live-high, train-low"), HV (high volume)

Table 8-2 depicts changes in serum hepcidin, MPO and LF throughout the three camps. Analysis of serum hepcidin concentration prior to and at the end of each individual camp highlighted increases of more than 25% above pre-camp values in every training camp, with the greatest increase (65%) observed at the end of the HI camp. Statistically significant changes however, were only observed in the LHTL camp, where post-camp samples had an average increase of 48% (p=0.001 ES=2.9) of pre-camp sample values. At the end of this same camp, LF concentration was significantly increased by 10% (p=0.008 ES=0.7). Serum concentration of MPO was increased by 4% above pre-camp after the HI camp, while a 21% and 11% decrease were observed at the end of the LHTL and HV camps, respectively. Data for IL-6 results are not shown as serum IL-6 values from both pre- and post-camp were all below the detection limit of the immune-assay (3.1pg/mL) for every training camp.

Table 8-2 – Iron and immune-related serum components pre- and post-camps

	Camp	Pre-camp			Post-camp			Mean % Change	t	p	ES
		Mean		SD	Mean		SD				
MPO (pmol/L)	HI	1191.2	±	525.1	1347.0	±	849.2	4.1	-1.03	0.343	0.23
	LHTL	704.0	±	464.9	551.6	±	590.0	-20.8	0.88	0.428	-0.19
	HV	515.5	±	306.0	459.0	±	279.1	-11.0	2.71	0.113	-0.19
Hepcidin (nmol/L)	HI	3.1	±	1.5	4.0	±	1.0	65.3	-1.55	0.173	0.74
	LHTL	3.3	±	0.6	4.9	±	0.5	48.1	-34.27	0.001*	2.92
	HV	4.3	±	0.7	5.3	±	0.2	25.5	-1.66	0.345	2.35
LF (nmol/L)	HI	25.7	±	5.4	23.9	±	6.8	-7.6	1.34	0.230	-0.29
	LHTL	16.8	±	4.8	20.5	±	5.7	10	-4.89	0.008*	0.70
	HV	18.0	±	4.9	18.1	±	3.0	2.5	-0.04	0.968	0.01

Paired t-test between pre- and post-camp samples. Degrees of freedom for each camp: HI (6), LHTL (4), HV (2) *Significant at $p<0.05$

Longitudinal comparison of all camps through linear mixed modelling indicated a significant effect of camp on serum MPO, LF and hepcidin. Values for MPO concentration obtained in the HI camp were significantly higher than those obtained in both the LHTL (p=0.012) and HV (p=0.005). Further analysis revealed that MPO concentration was significantly higher at the end of the HI camp (1347±849 pmol/L) compared to both the LHTL (551.6±590 pmol/L p=0.026) and HV (459±279 pmol/L p=0.024) camps. Serum concentration of LF also fluctuated between the camps, with higher concentrations found in the HI, compared to the LHTL (p=0.006) and HV (p=0.036) camps. Pairwise comparison, derived from the mixed model, between pre-camp samples of the three camps showed that values for LF obtained prior to the HI camp were 35% and 30% higher than pre-camp values for both the LHTL (p=0.004) and HV (p=0.067), respectively (Table 2). The linear mixed model's test of fixed effects revealed the significance of both 'time' (p=0.002) and 'camp' (p=0.023) in the outcome of hepcidin concentration. In this model it was clear that the LHTL and HV camps had a significant effect on hepcidin concentration and that the training itself increased hepcidin concentrations, as time (pre- *versus* post-camp) was a significant effect in the model.

Discussion

The main findings of this study were the increase in circulating erythrocyte-related parameters following the LHTL camp compared to the HI and HV camp. The increase in the percentage of neutrophils in circulation from pre- to post-camp was observed in the LHTL but not in HI or the HV camps. In contrast, the percentage of circulating lymphocytes decreased post-camp compared to pre- LHTL camp values, while an increase in circulation for this population was observed post-HI and HV camp. HI camp caused an increase in serum levels of MPO. While the LHTL camp caused an increase in serum levels of LF, the HI and the HV camps did not seem to affect serum levels of LF. Additionally, this study demonstrated that serum hepcidin concentration is responsive to the training stimulus (i.e. intensity) and that such a response is augmented as a hypoxic challenge is added to the physical stress.

The concentrations of erythrocytes and haemoglobin were significantly increased at the end of the LHTL camp, contrary to the trend observed in both the HI an HV camps (Figure 8-3a-c). It is well established that hypoxic exposure triggers erythropoiesis, mainly through release of EPO, despite studies adopting the LHTL protocol being unable to produce consistent results in erythrocyte-related parameters, mostly due to the lack of standardisation in the hypoxic component (simulated or not) and/or duration of the exposure (Ashenden, Gore, Dobson, & Hahn, 1999; Levine & Stray-Gundersen, 2005; Rusko, Tikkanen, & Peltonen, 2004; Wehrlin et al., 2006). Recently, Alvarez-Martin et al. (2016) demonstrated the effects of hypoxia on the bone-marrow micro-environment, utilizing a clinically-relevant intermittent hypoxia rat model for 30-35 days, mimicking effects of obstructive sleeping apnoea syndrome (OSA). The OSA model restricts O_2 delivery throughout the night (~10.5h/day), similar to the hypoxic exposure the athletes underwent during the LHTL camp. This group demonstrated an increase in erythrocyte colonies (specifically burst forming unit-erythroid) in the bone-marrow as well as increases in circulating erythrocyte concentration in rats exposed to hypoxia compared to their normoxia-exposed counterparts. These results, as well as others specifically reporting increases in erythrocyte-related parameters in elite athletes exposed to the LHTL protocol (Bonetti, Hopkins, & Kilding, 2006; Brugniaux et al., 2006; Siebenmann et al., 2012), are in agreement with results shown in this study.

While LHTL studies reporting thrombocyte concentrations are scarce, this study has shown that such a protocol may lead to an increased concentration of thrombocytes in peripheral blood. It is reasonable to suggest that hypoxia may stimulate differentiation of megakaryocyte progenitors in the bone marrow, as observed in rats exposed to hypoxic chambers with reduced air pressure (~307.5mmHg) for 6 hours (Lebedeva, Yushkov, & Chereshnev, 2003). However, it is not confirmed that such an increase in bone marrow megakaryocytopoiesis is reflected in increases in platelet concentrations in peripheral blood. Further investigation with human subjects is required.

The most significant change in the peripheral distribution of neutrophils in blood obtained from pre- to post-camp was observed after the LHTL camp, where there was as 20.7% increase in the percentage of circulating neutrophils. Interestingly, this was different to the HI and HV camp where post-camp percentages of circulating neutrophils were lower than pre-camp values. The concentration and percentage of circulating neutrophils in the HV camp, in both pre- ($2.4x10^3$/µL; 45%) and post-camp ($2.3x10^3$/µL; 42.8%) measurements, were the lowest recorded for all camps. Peripheral lymphocyte distribution, however, behaved opposite to that, with circulating percentages decreased at the end of the LHTL camp, but increased after the HI and HV camps. The previously mentioned study by Alvarez-Martin et al. (2016) demonstrated an upregulation of myeloid progenitor cells and B lymphocytes and a significant decrease in the percentage of T lymphocytes in the bone marrow of hypoxia-exposed rats compared to the control animals kept in normoxia. As T lymphocytes represent 61-85% and B lymphocytes only 7-23% of total lymphocytes (Reichert et al., 1991), changes to the T lymphocyte subset would presumably dictate the trend observed. Most interestingly is the finding that such changes in haematopoietic lineages were reflected in peripheral blood (Alvarez-Martins et al., 2016), in agreement with findings obtained from peripheral blood in this study. This may lead to the conclusion that while HI and HV training camps apparently favour the proliferation of cells mediating the adaptive immunity (i.e. lymphocytes), the additional stimuli provided by the LHTL camp enhances circulation of myeloid-derived innate-immunity components.

Concomitantly to the increase in circulating neutrophils in the LHTL camp, there was a significant increase in serum concentration of LF. The origin of serum lactoferrin is mostly from neutrophil degranulation (Iyer & Lonnerdal, 1993). In an elegant *in-vitro* study on the effects of hypoxia (0.8% O_2 and 0.5% CO_2) on neutrophilic degranulation,

Honderdos (2014) demonstrated a significant increase in specific granule exocytosis, quantified by an increase in lactoferrin concentration of 240% and 250% in the supernatant of freshly isolated neutrophils (human) stimulated with GM-CSF + fMLP and cytochalasin-B + fMLP, respectively. It is not fully understood how hypoxia, as experienced locally through inflammation or systemically through, or, in the context of this thesis, the LHTL training camp, triggers degranulation in peripheral neutrophils. There are suggestions that hypoxia causes a rearrangement of the neutrophilic cytoskeleton (Honderdos, 2014), which in turn may signal down-stream molecules such as GTPases Rac and Rho, which are involved in the PI3K cascade (Akasaki, Koga, & Sumimoto, 1999; Welch, Coadwell, Stephens, & Hawkins, 2003) and are vital for azurophilic granule exocytosis (Rac2) (Abdel-Latif, Steward, & MacDonald, 2004; Eitzen et al., 2011). Hoenderdos (2014) has shown that PI3Kγ is crucial in the signalling events that lead to the increased degranulation observed during hypoxia. Further, McGovern et al. (2011) demonstrated that while hypoxia did not cause degranulation (measured through elastase release) in unstimulated neutrophils, elastase release from cytochalasin-B–primed fMLP-stimulated neutrophils increased significantly. It is reasonable to believe that the combined effects of training-induced APR (via increase in IL-6, TNF-α) and the hypoxic stimulus during the LHTL camp may mimic events in an inflammatory site, where O_2 availability is diminished and neutrophils are primed (i.e. enhanced phagocytosis and ROS production; Condliffe et al. 1998), thus increasing serum LF as an immune strategy to ensure any available iron is not used by proliferating pathogens.

The high variability of serum hepcidin concentration observed in the HI camp (i.e. large standard deviations) demonstrates the individual-specific responsiveness of hepcidin. In this camp, two athletes presented 2.5 and 3.5-fold increases from pre- to post-camp values. Individual changes of these magnitudes were not observed in either the LHTL or HV camps. Curiously, these athletes were not further selected to participate on the LHTL and HV camps. The increase in serum hepcidin concentration at the end of every training camp demonstrates the effect that physical exertion has on hepcidin concentration, as shown previously by others (Auersperger et al., 2013; Peeling et al., 2009a; Skarpanska-Stejnborn et al., 2015). Interestingly, the LHTL camp, despite a lower percent change from pre- to post-camp compared to the HI camp, still had a significant 48% increase in hepcidin concentration. The hypoxic challenge is known to negatively regulate hepcidin expression through signalling pathways including HIF-α, platelet-derived growth factor

(which blocks cAMP response-element binding protein/H; Sonnweber et al., 2014) and EPO (Kautz & Nemeth, 2014; Rishi et al., 2015). The hypoxic stimulus results in EPO release for increased RBC production. Erythroid precursors then express erythroferrone (EFRE) which down-regulates hepcidin to allow iron to be absorbed and distributed to the bone marrow for erythropoiesis (Kautz et al., 2014). This stimulus occurs at the onset of exposure to hypoxia. Possibly the increase in hepcidin expression post- LHTL camp demonstrated a feed-back regulation where sufficient iron had been absorbed and delivered to the bone marrow and at the time of testing hepcidin was being up-regulated to re-establish iron homeostasis as athletes were taking iron supplements. It may be possible that the expected down-regulation of hepcidin occurred during the acclimatisation phase of the LHTL camp, which happened one week before the pre-camp samples were obtained from the athletes. Goetze et al. (2013) described an increase in established erythropoietic markers (i.e. EPO and GDF-15) after two days of arrival at 4559m and at the same time a significant decline in hepcidin levels, which reached lowest values after four days. Using the same methodology of hypoxic exposure as used in this thesis (i.e. LHTL), Govus et al. (2016) characterized the hepcidin response prior to altitude exposure (baseline) and on days two and 14 of LHTL. This research group demonstrated a significant decrease in plasma hepcidin after two days of exposure compared to baseline, which, despite a trend towards returning to baseline values, the decrease was still significant after 14 days. Further, if erythropoiesis was occurring after the camp it would most likely be reflected in a decrease in serum iron concentration at the same time-point (Goetze et al., 2013). In contrast, serum iron concentration was increased by 15% from pre-camp values at the end of the LHTL camp. It has been proposed that the molecular signalling for hepcidin expression may be more responsive to iron status/stores (BMP/SMAD pathway) than to hypoxia (Huang, Constante, Layoun, & Santos, 2009); therefore, adequate or increased iron availability would trigger the up-regulation of hepcidin expression to maintain iron homeostasis, even in the presence of the hypoxic stimulus. Findings from the previously mentioned study undertaken by Govus et al. (2016) may also aid in understanding the interactions between training induced inflammation, hypoxia, hepcidin and iron supplementation, as in their study, iron-supplemented athletes did not show an up-regulation of hepcidin after 14-days of LHTL exposure as did the non-supplemented athletes.

The slight decrease in resting plasma ferritin concentration after every camp may be related to the training stimulus imposed. After the HI training camp, ferritin levels were decreased by 3.4% of baseline value, while after the HV training camp, this decrease was 2.5%. While there is an established positive correlation between serum ferritin and training load (Koehler et al., 2012; Malczewska et al., 2000), correlations with training volumes are poor (Ostojic & Ahmetovic, 2008). Interestingly, a 3.0% decrease from pre-camp values was observed at the end of the LHTL camp. In this camp, the altitude challenge, combined with the training sessions, had a greater imposition on erythrocyte production, as observed by a significant increase in erythrocyte parameters at the end of this camp, compared to the other two camps (Figure 8-3). Govus (2015) reported a 33% reduction from baseline in serum ferritin concentration in elite athletes after moderate exposure (1,500-3,000m) for 21±3 days without iron supplementation. Curiously, ferritin concentration from iron-supplemented athletes only decreased by 13% from baseline values (Govus, 2015). As declines in ferritin observed after the LHTL camp are comparable to those of the HI and HV camps, it may be concluded that by providing iron-supplementation to the athletes in this study, iron stores were maintained during the hypoxia-driven increase in iron requirement for erythropoiesis, as demonstrated by others (Dellavalle, 2013; Govus, 2015). The 16% increase in serum iron observed at the end of this camp, but not at the end of the other two camps, supports such hypothesis.

Training-induced perturbation of immune and iron-related parameters may lead to reduction in performance and increased susceptibility to infections. This study demonstrated that such disturbances are training-period specific, enabling coaches to better structure the training periods to avoid potentially negative changes in immunological capacity and iron status that may impact performance. Future studies should aim to establish if there is a hierarchy in the signalling pathways involved in hepcidin mRNA expression. If an 'over-riding' mechanism exists, the exercise-induced anaemia of inflammation may be attenuated, as it could be counter-acted with hypoxic stimuli combined with iron supplementation. By avoiding decrements in functionally available iron and iron stores ideal erythro- and myelopoiesis can be ensured. In a cross-over study, Badenhorst et al. (2014) demonstrated that three hours of hypoxic exposure (F_IO_2 ~15%) during the recovery phase post-interval running session (8 × 3 minutes repeats at 85% $\dot{V}O_{2peak}$ with 90 seconds of active recovery (60% $\dot{V}O_{2peak}$) between repetitions) attenuated circulating hepcidin concentrations in male endurance runners compared to their recovery in normoxia. This not only solidifies the suggestion made

previously but paves the way for further investigation into attenuating training-induced acute-phase response in order to maintain functionally available iron and consequently aid recovery.

Chapter 9

Discussion

This set of studies provides unique insight into the physiological/immunological processes induced by a range of training regimes in elite female kayak athletes in preparation for, and while endeavouring to gain selection in the team to compete in, the 2016 Rio Olympic Games. Throughout the training year (conducted during 2015), the effects of the training camps organised by AIS were studied based on the different training methodologies employed. Using a micro-sampling technique exclusively developed for this thesis, data on immune function and iron status were collected from three separate training camps: HI, LHTL, and HV. Performance outcomes after each camp were measured by the success of the athlete at the subsequent competition, most of which were international championships. Results from each event determined the continuation of the athlete in the Olympic training program. Main findings were:

- The LHTL camp (18 days) enhanced the circulation and function of granulocytes compared to both the HI (2 weeks) and the HV (2 weeks) training camps

- Improved recovery from the post-exercise decrease in neutrophilic stimulated ROS production was observed in the LHTL camp compared to the HI and HV camp.

- The HV training camp promoted circulation of lymphocytes while the LHTL promoted increased circulation of neutrophils.

- As expected, the LHTL camp, combined with iron supplementation, increased erythrocyte, haemoglobin, haematocrit, and thrombocyte concentration while, in contrast, the HI and HV camps caused decrements in these parameters.

- The introduction of normobaric hypoxia (via LHTL) and iron supplementation to a HI training camp partially suppressed the post-camp resting serum hepcidin concentration.

It was demonstrated that during different training periods, post-training leucocyte distribution is dictated by the intensity and duration of the daily training imposed. Additionally, this study highlighted the training-dependent modulation of the immune capacity through changes in the expression of surface receptors of leucocytes. Such

receptors not only identify specific leucocyte sub-populations but relate to specific leucocytic functions. Similarly, granulocytic functions of phagocytosis and stimulated oxidative burst are shown here to be affected not only by daily training load but also by the accumulation of such loads in periods of HI, LHTL and HV training.

Throughout the different training camps, lymphocyte subsets showed exercise-induced redistribution from pre- to post-training. These acute responses have been described extensively in the literature (Campbell et al., 2009; Fry, Morton, & Keast, 1992; Gleeson, Bishop, Oliveira, McCauley, & Tauler, 2011; Nieman et al., 1994; Tvede, Kappel, Halkjaer-Kristensen, Galbo, & Pedersen, 1993), with NK and B lymphocyte subsets demonstrating the greatest mobilization, compared to helper and cytotoxic T lymphocytes. Amongst the training camps analysed, the HI training camp caused the greatest mobilization from pre- to post-training of these subsets. Interestingly, even though the LHTL camp imposed large training load and provided large adaptive stimulus, the redistribution of B lymphocytes throughout the LHTL did not mimic the HI camp as did the NK lymphocytes. This original finding brings into the question the impact of altitude training on mobilisation of adaptive components of the immune system as the expected acute B- lymphocyte redistribution from pre- to post-training (Morgado et al., 2014; Navalta et al., 2013; Turner et al., 2016) was abolished during LHTL, maintaining B-lymphocyte concentration constant throughout the training camp. It may be the case that the paucity in B-lymphocyte recruitment to the circulation during the LHTL camp demonstrates a preferred recruitment of innate rather than adaptive immune components during hypoxic challenges. Hypoxia, as observed in the inflamed tissue, promotes the circulation and migration of neutrophils towards the affected tissue (Sica, Melillo, & Varesio, 2011), which strongly supports the findings of this study.

Neutrophils have been erroneously portrayed as a short-lived, non-proliferative, non-specific type of leucocyte. Its highly condensed nucleus added to the notion of a terminally differentiated leucocyte thought not to perform gene expression. Such assumptions have been refuted as neutrophils are known to synthesize heat-shock proteins though a transcription-dependent manner (Eid, Kravath, & Lanks, 1987) and to regulate RNA synthesis and gene expression in response to various stimuli (Zhang et al., 2004). Neutrophils also orchestrate the inflammatory response through up-regulation of genes encoding for chemokines and cytokines such as TNF-a, IL-1b, IL-1ra, IL-8 (McDonald, Bald, & Cassatella, 1997). More specific to the functions analysed in this thesis,

neutrophils express mRNA-encoding phagocytic receptors (e.g. FcR; Jack and Fearon, 1988) and up-regulate the genes encoding the NADPH oxidase cytochrome components $gp91^{phox}$ and $p22^{phox}$ in response to surface antigen coupling with specific cytokines (e.g. TNF-α and GM-CSF) and bacterial LPS (Newburger, Dai, & Whitney, 1991; Newburger, Ezekowitz, Whitney, Wright, & Orkin, 1988). The reduced number of mitochondria and amount of mitochondrial respiration compared to other leucocytes, led researchers to believe that such organelles did not play a role in neutrophilic functions. However, it is now known that, contrary to other leucocyte populations, the mitochondria in neutrophils is displayed as a tubular network and they are involved in functions such as chemotaxis and apoptosis (Bao et al., 2015; Maianski et al., 2004). Curiously, both these functions require cytoplasmic rearrangement. Description of neutrophilic behaviour during surface antigen-dependent activities such as rolling, tethering and adhering to the vascular epithelium demonstrate clear remodelling of the cytoplasm, which may also indicate mitochondrial involvement (via ATP; Bao et al., 2014). Fossati et al. (2003) demonstrated that disruption to the neutrophilic mitochondrial membrane potential perturbed structural rearrangement of the cytoplasm, heavily influencing neutrophilic morphology. It is known that under physiological shear flow, neutrophils project pseudopods increasing the contact area with the epithelium enabling firm adhesion which precedes successful chemotaxis (Rocheleau, Sumagin, Sarelius, & King, 2015). Therefore, the involvement of the mitochondria in the multitude of functions described indicates that neutrophils may have a functional-dependent requirement for iron possibly to be incorporated to the ETC, haem and/or Fe-S clusters. The identification of receptors for transferrin on the neutrophilic surface (Maneva & Taleva, 2009) cements such a suggestion. In the LHTL camp, the acute post-training up-regulation of transferrin receptors, accompanied by increases in CD11b/CD18 complex on the granulocytic surface, is consistent with an increased demand for iron for cellular activation.

The importance of maintaining a functionally-available iron supply during training periods undoubtedly exceeds the oxygen-carrying and erythropoietic functions of iron. Immune competence throughout these highly demanding training periods is challenged due to alterations in iron availability, as lymphocytes, monocytes, and granulocytes - particularly made evident in this thesis - have a functional-related demand for iron. Any limitation in the availability of iron has the potential to compromise immune function via a decrease in ATP produced (secondary to impairment of oxidative phosphorylation) and/or via a range of other iron-dependent processes critical in leucocytes (e.g.

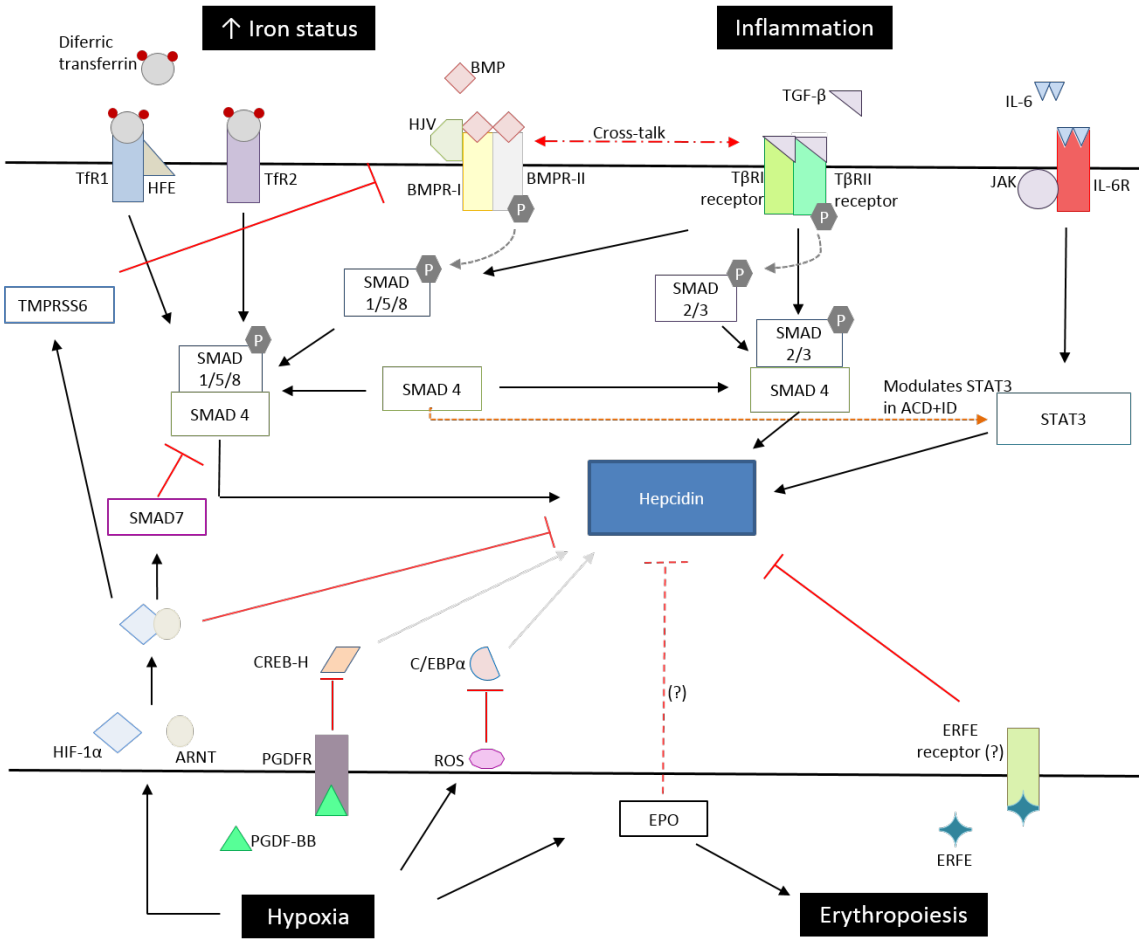
lymphocyte proliferation). The noteworthy increase in stimulated oxidative burst capacity observed on the last testing day of the LHTL camp may have been a combination of increased exposure to iron (supplement) (Chandra, 1973, 1976, 1979; Chandra & Saraya, 1975; Dallman, 1986; Moore & Humbert, 1984; Yetgin, Altay, Ciliv, & Laleli, 1979) and training-induced delayed neutrophilic apoptosis (Mooren et al., 2012). The post-exercise increase in CD71 expression (discussed above) and the increase in oxidative burst capacity observed in samples obtained hours later (prior to training) are chronologically in agreement with the time lag for the translation and synthesis of NADPH oxidase components, which require iron incorporation (Newburger et al., 1991). The increased time neutrophils are in the circulation due to the delayed apoptosis extends neutrophilic exposure to plasma cytokines and LPS, known to be increased post-exercise (Ashton et al., 2003; Bernecker et al., 2013; Pedersen & Toft, 2000; Starkie, Rolland, Angus, Anderson, & Febbraio, 2001), particularly if considering the possible cumulative effects of multiple training sessions per day. LPS-induced neutrophilic activation is mediated by a cluster of receptors including the toll-like receptor 4 (TRL4), CD14 and CD11b/CD18 (Kabanov, Grachev, & Prokhorenko, 2014; Schymeinsky, Mocsai, & Walzog, 2007; Wright, Ramos, Tobias, Ulevitch, & Mathison, 1990; Zarewych, Kindzelskii, Todd, & Petty, 1996). Through two distinct pathways (myeloid differentiation primary response gene 88 (MyD88)-dependent and MyD88-independent), mitogen activated protein kinase (MAPK) and interferon regulatory factor 3 are activated, respectively, translocating NF-κB. Both signalling pathways are involved in priming of the neutrophil oxidative burst, through increase membrane expression of flavocytochrome b_{558} via exocytosis of specific and gelatinase granules as well as secretory vesicles (Ward, Nakamura, & McLeish, 2000). Interestingly, GM-CSF and TNF-α, which are both found in higher concentrations in plasma post-exercise, also prime neutrophilic oxidative burst via the MAPK pathway (McDonald et al., 1997; McLeish et al., 1998; Suzuki, Hino, Hato, Tatsumi, & Kitagawa, 1999).

Findings in this thesis support the acute post-exercise serum hepcidin up-regulation previously discussed by others (Govus et al., 2016; McClung et al., 2013; Peeling et al., 2009b; Peeling et al., 2014; Sim et al., 2012). Further, findings of this research indicate that resting serum hepcidin concentration is dependent on not only training intensity, but also hypoxic exposure. Such results raise questions about the regulatory role and the possible signalling-hierarchy that inflammation, iron status and hypoxia have on iron regulation. Signalling of iron status and inflammation (IL-6) through the SMAD and

STAT3 pathways, respectively, upregulate hepcidin expression. In contrast, it is well established that hypoxia, through EPO, HIF-1α or platelet-derived growth factor receptor/cAMP response-element binding protein/H pathways, is a negative regulator of hepcidin expression. EPO-dependent erythropoiesis leads to activation of ERFE pathway (Kautz et al., 2014) and the activation or stabilization of matriptase-2 (transmembrane serine proteinase TMPRSS6) which inhibits the hepcidin-upregulating BMP-SMAD signalling pathway (via cleavage of HJV) (Nai et al., 2016). Here it was observed that hepcidin expression was enhanced after a HI training camp, in line with previous findings which associate hepcidin up-regulation to IL-6 increase post-exercise (via JAK/STAT3 pathway). Training not only increases circulating levels of IL-6 in plasma but also other cytokines such TGF-1β (Czarkowska-Paczek, Bartlomiejczyk, & Przybylski, 2006). This superfamily of cytokines has been shown to signal SMAD1/5/8 and SMAD2/3 complexing with SMAD4. These signalling pathways and SMAD1/5/8 + SMAD4 complex also up-regulates hepcidin expression through BMP6 signalling, suggesting an additional 'inflammation-induced' avenue for hepcidin up-regulation. Recently it has been shown that hypoxia induces HIF-1α stabilization and consequently expression of genes regulated by this transcription factor, such as Glut1. HIF-1α has been suggested to directly inhibit hepcidin expression. Hypoxia causes an up-regulation of SMAD7 in endothelial cells (Chi et al., 2006), and most recently such up-regulation has been shown to be HIF-1α dependent (Heikkinen et al., 2010). SMAD7 is an antagonist of TGF-β and BMP signalling through negative-feedback mechanisms (Guo & Wang, 2009; Yan & Chen, 2011; Yan, Liu, & Chen, 2009). Recently Nai et al. (2016) highlighted the importance of inhibition of the BMP-SMAD signalling pathway for effective ERFE-driven hepcidin down-regulation. Therefore, as proposed in Figure 9-1 (below), while HI exercise triggers hepcidin up-regulation, possibly through STAT3 and SMAD signalling, hypoxia-induced increase in ERFE and HIF-1 activation of SMAD7 may be down-regulating hepcidin expression. The combination of HI exercise and hypoxia in this study demonstrated a 26.3% down-regulation of post-camp resting serum hepcidin compared to HI alone. But, resting hepcidin values post-LHTL were still significantly increased from baseline values, indicating that the hepcidin-promoting signalling was greater than that of the hypoxia-induced down-regulation under the specific experimental conditions of the LHTL study. Further, a 'cross-talk' between the BMP/SMAD and other pathways has been established (Guo & Wang, 2009) highlighting that iron status may modulate other pathways, as SMAD4 modulates STAT3 activity in ACD with ID (Theurl et al., 2011). This leads to two important observations: (1) maintenance of iron status is vital

for hepcidin regulation and (2) iron availability for erythropoiesis will determine hepcidin expression if opposing signalling pathways are activated.

Figure 9-1 – Model of hepcidin regulation via inflammation and hypoxia



Exercise induced hepcidin regulation, suggested to occur via STAT3 and SMAD signalling pathways, may be counteracted by the inclusion of a hypoxic challenge. Hypoxia down-regulates hepcidin expression via SMAD7 pathway. Iron status, through SMAD4, may regulate other pathways (i.e. STAT3). BMP, bone morphogenetic protein; BMPR-I, BMP receptor-I; BMPR-II, BPM receptor-II; C/EBPα, CCAAT/enhancer-binding protein alpha; CREB/H, cAMP response-element binding protein/H; EPO, erythropoietin; ERFE, erythroferrone; HFE, hemochromatosis protein; HIF, hypoxia-inducible factor; HJV, hemojuvelin; IL6, interleukin 6; IL-6R, interleukin 6 receptor; JAK, Janus kinase; PDGF-BB, platelet-derived growth factor-BB; PDGFR, platelet-derived growth factor receptor; SMAD1/5/8, sma and mothers against decapentaplegic homologue 1/5/8 complex; SMAD2/3, SMAD homologue 2/3; SMAD4, SMAD homologue 4; SMAD7,SMAD homologue 7; STAT3, signal transducer and activator of transcription 3; TFR1, transferrin receptor 1; TFR2, transferrin receptor 2. *Adapted from Huang et al. (2009) and Rishi et al. (2015).*

As the highly demanding training load was sustained from the HI to the LHTL camp (2502±187AU and 2579±265AU, respectively), the altitude challenge and the iron supplementation were the main factors distinguishing both camps. Given the pathways that regulate hepcidin expression described above, findings of this thesis are in accordance with recent findings of decreased serum hepcidin concentration after hypoxic

stimulus - both short-term (3 hours) simulated hypoxia interventions (F_IO_2 ~ 15%) and 14-days LHTL (11h/day at F_IO_2 ~ 15%) - (Badenhorst et al., 2014; Govus et al., 2016). It is possible that iron supplementation prevented the 4% reduction in ferritin observed post-HI, from occurring post-LHTL. Recently, Garvican-Lewis et al. (2016) highlighted that iron-supplementation (105mg/day) during LHTL, even in iron-replete athletes (ferritin>100ug/L), was beneficial for the sought haematological outcome (i.e. increased Hb_{mass}). As both the concentration of serum iron and transferrin saturation were higher at the end of LHTL compared to post-HI camp, it is reasonable to suggest that the use of iron supplementation acted in maintaining and even increasing iron availability. The results shown in this thesis demonstrate that such supplementation during the hypoxic challenge may also benefit innate immune responses.

Interestingly, the increase in serum LF post-LHTL was not observed post-HI, which may lead to the suggestion that the hypoxic challenge itself was contributing to the degranulation of granulocytes (Sica et al., 2011). It has been established that hypoxia causes increased permeability and production of CXCL8 and other chemokines of endothelial and epithelial cells enhancing neutrophilic adhesion and migration (Arnould, Michiels, Janssens, Delaive, & Remacle, 1995; Colgan, Dzus, & Parkos, 1996; Rainger, Fisher, Shearman, & Nash, 1995). Additionally, hypoxia increases serum IL-6 concentration (Hartmann et al., 2000; Klausen et al. 1997; Ertel et al. 1995). Therefore, it is possible that an additive effect of the exercise-induced and the hypoxia-induced 'inflammation' may have increased the hepcidin response seen at the end of the LHTL camp. Further studies are required to elucidate a dosage of hypoxic exposure that would promote immune capacity while still maintaining the desired performance-related outcomes. Resting hepcidin concentration was also increased post-HV camp, but not to the same magnitude as in both the HI and LHTL camps. Based on this novel longitudinal observation, it is proposed that the adoption of a greater volume (and reduced intensity) of training during an iron-supplemented LHTL intervention may further attenuate the resting post-camp hepcidin concentration thereby maintaining functionally available iron. The year-long investigation of the response of Olympic level athletes to specific training periods (HI, LHTL, HV) provides new insights into the interaction between training load, iron-metabolism and immune function. Such insights require a re-evaluation of what has been identified as the potentially negative consequences of training induced inflammation (i.e. limiting erythropoiesis and iron-related responses) when superimposed on a period of LHTL training. Maintenance of iron stores and readily available iron during HI training

periods is vital for adequate innate immune function. The introduction of a hypoxic challenge, combined with iron supplementation, may promote innate immune competence, thus increasing the opportunity for hypoxia-based recovery protocols to be suggested.

In summary, this thesis describes a connected series of studies across a full training year involving elite level female kayak athletes preparing for the 2016 Rio Olympic Games. It has established firstly, that specific training interventions (i.e. HI, LHTL, HV) differentially affect both the immune and haematological systems, and suggests that training prescription should now consider the effects that perturbations to such systems may have on performance and desired training adaptations.

Secondly, it has established that the inflammatory response induced as part of HI training may interfere with the haematological response to hypoxia (e.g. increased haemoglobin concentration), thus potentially limiting adaptation when inflammation is superimposed on the stimulus provided by a LHTL protocol.

Thirdly, the leucocyte composition of blood and the functional characteristics/capacities of specific leucocyte subsets (e.g. phagocytosis, receptor expression) varies as a function of tissue location and hence sampling site. Samples drawn from different sites can no longer be considered to be identical and should not be used interchangeably.

Limitations

Although the results of this thesis have practical applications and the potential to aid in maintenance of immune competence in elite female athletes through different training periods, as adopted in preparation for the Olympic Games, the following limitations do apply:

- The sampling times were restricted and did not allow a more extensive post-camp evaluation of both immune and iron status since most athletes involved in the camps were interstate.

- Evaluation of immune and iron status could not be performed throughout a tapering period as all tapers in 2015 were held at overseas training camps.

- All results obtained are limited to a specific population of elite female kayak athletes with resting pre-camp ferritin levels greater than 90pmol/L (40µg/L), Hb levels greater than 130g/L and transferrin saturation above 20%, indicative of normal iron stores.

- The small number of senior elite athletes chosen for the 2016 Olympic games did not allow for distinctive analysis between eumenorrheic and oral contraceptive pill users. Sim et al. (2015) demonstrated that oral contraception does not alter the post-exercise hepcidin response, however, it is not yet established if resting hepcidin values are altered, particularly with a LHTL intervention.

Practical Applications

The findings of thesis may translate into practical recommendations regarding athletic monitoring and prescription of training for elite athletes as follow:

- The blood microsampling technique presented herein allows monitoring of cellular immune function and phenotypical distribution of leucocytes in circulation.
- The transient innate immune suppression observed after intense training camps is attenuated as the athlete is exposed to periods of hypoxia, as adopted in the LHTL camps.

- Iron supplementation throughout the LHTL camp not only ensures that iron status is maintained for erythropoiesis but also for adequate immune cell function.

Future Research

- Establish hepcidin responses in iron-deficient athletes undergoing LHTL. Future studies should aim to describe the kinetics of the hepcidin response in the acclimatization phase as well as the training phase.

- Determine if LHTL without iron supplementation for iron replete athletes impacts granulocytic functions.

- Implement multiple LHTL training camps per season in female kayak athletes, to establish if there is an exposure-threshold for the down-regulation of hepcidin through hypoxia as research has shown that LHTL induces higher HIF-1a mRNA sensitivity to acute hypoxia (Pialoux et al., 2009). Garvican-Lewis et al. (2013) and Saunders et al have shown success in multiple altitude exposures throughout the preparation of water polo and race walking, respectively, for the 2012 Olympic games. Saunders et al. 2003 has shown improvement in 400m events, which would be physiologically more similar to the demands experienced by sprint kayak athletes. Future research should aim to demonstrate the possible immunological benefits of multiple LHTL training camps.

- Determine clinical and performance consequences of monitoring and managing immune competence and iron status throughout different training periods. Future research should aim to incorporate a control group to quantify more precisely the independent effects of training, hypoxia interventions and iron supplementation.

- Aim to further characterise signalling pathways linking iron availability, inflammation, and hypoxia to hepcidin expression.

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Appendix

August, 2013

Haematological, Biochemical and Immunological Markers of Immune Function In Elite Athletes during Different Training Periods.

BUHREC PROTOCOL NUMBER: RO1721

BOND UNIVERSITY
BRINGING AMBITION TO LIFE

FACULTY OF HEALTH SCIENCES AND MEDICINE

Bond University
Gold Coast, Queensland 4229
Australia

Toll free 1800 753 855
(within Australia)

Ph: +61 7 5595 4400
Fax: +61 7 5595 4122
(from overseas)

Email: hsm@bond.edu.au

ABN 88 010 694 121
CRICOS CODE 000017B

HAEMATOLOGICAL, BIOCHEMICAL AND IMMUNOLOGICAL MARKERS OF IMMUNE FUNCTION IN ELITE ATHLETES DURING DIFFERENT TRAINING PERIODS.

My name is Elisa Canetti and I am currently completing a PhD at Bond University under the supervision of Associate Professor Dr. Bon Gray.

I am conducting a research investigation into haematological, biochemical and immunological markers of immune function. I am specifically interested in the influence of different training modalities on iron and immune function related parameters.

I am seeking healthy male and female students or staff from Bond University and elite athletes between 18 to 35 years of age. This study will provide participants with insight on their health and well-being in regard to different training modalities, through iron metabolism and immune function parameters. This will benefit individual optimal performance level as participants will be able to correlate their training demands with their health and therefore aim to avoid overtraining and minimise illness during crucial training periods or competition. As part of this study, I will invite you to keep a health log identifying any relevant health related issue that you may experience during the study. In order to identify possible markers of immune function, a venous and capillary blood sample will be requested from you at different moments of the research – based on training schedule of athletes involved in the study. Blood collection will cause no disruption to normal training procedures. The samples should not exceed 50mL (venous) or 500µL (capillary) on a single draw, and should not be taken in consecutive days.

Participation in this study is completely voluntary and you may withdraw at any time without risking any negative consequences. If you choose to withdraw your participation in this study, the information you have provided will be immediately destroyed. All the data collected in this study will be treated with complete confidentiality and not made accessible to any person outside of the researchers working on this project. The information I obtain from you will be dealt with in a manner that ensures you remain anonymous. Data will be stored in a secured location at Bond University for a period of 5 years in accordance with the guidelines set out by the Bond University Human Research Ethics Committee.

It is anticipated that the data collected during this study will assist us in understanding the influence of different training modalities on immune function.

Your participation in this study will enhance work towards finding possible predictive markers of immune function.

If you experience distress from participation in this research, please contact:

Elisa Canetti (Student Investigator)
elisa.canetti@student.bond.edu.au

Dr. Bon Gray (Supervisor)
bgray@bond.edu.au
+61 7 559 54454

Should you have any complaints concerning the manner in which this research is being conducted please make contact with:
Bond University Human Research Ethics Committee,
c/o Bond University Office of Research Services.
Bond University, Gold Coast, 4229
Tel: +61 7 5595 4194
Fax: +61 7 5595 1120
Email: buhrec@bond.edu.au

We thank you for taking the time to assist us with this research.

Yours sincerely,

Elisa Canetti

Dr. Bon Gray

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